

**FLAVOUR DEVELOPMENT OF EAST MIDLANDS
CHEESES AND EVALUATION OF FLAVOUR
PRODUCING MICROORGANISMS IN A SMALL SCALE
REAL-CHEESE MODEL**

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ABSTRACT

Stilton is a blue-veined cheese made from pasteurised milk. The diversity of the microflora found within the cheese helps develop the unique flavour and aroma of Stilton compared to other blue cheese. However, this flora is not controlled and so product may be variable. A small-scale cheese model was developed to allow examination of the effect of different microflora on flavour production in a controlled way.

Texture analysis, water activity and viable count of the cheese models were compared to commercial cheeses in order to optimise the model.

The final model had the hardness of Danish Blue and the water activity of Stilton. The growth of lactic acid bacteria through the cheese making process was monitored and showed a characteristic reduction after salting. Changes in total aerobic count and yeast and moulds after salt addition and handling showed the ease with which the cheese can become contaminated with external flora such as yeasts and moulds even under aseptically manipulated conditions.

Solid Phase Microextraction Gas Chromatography Mass-Spectrometry was used to evaluate the flavour volatiles produced when milk and cheese were inoculated with different microorganisms. Some yeast species such as *Trichosporon beigleii* inhibited the metabolism of *P. roqueforti* within the models, whereas others such as *Debaryomyces hansenii* aided the metabolism of compounds in the models to develop different flavour volatiles.

Overall a small-scale blue cheese model was produced and can be used to understand the role of different organisms in the development of the flavour and aroma of blue cheeses in the future.

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1 INTRODUCTION

1.1 STILTON

1.1.1 DESCRIPTION OF STILTON

There are two varieties of Stilton, “Blue Stilton” and “White Stilton.” Both carry ‘EU Protected Designation of Origin’ status and can only be produced in the East Midlands of the UK, specifically Nottinghamshire, Leicestershire and Derbyshire. The cheese itself is un-pressed, left to develop its own crust or coat and the blue variety is internally mould-ripened.

White Stilton is a young, crumbly cheese which breaks down in the mouth giving a creamy finish. Good Blue Stilton is semi-soft with a creamy texture and has an even distribution of blue veins formed by internal growth of *Penicillium roqueforti*. It has a creamy flavour to it, but other flavours vary between different producers, and even between batches of the same producer. This is due to the complex production of blue cheese in general and in particular Stilton (LCD, 2012).

1.1.2 HISTORY OF STILTON

Stilton was first produced in the early 18th Century from the English county of Leicestershire. The cheese takes its name from Stilton village in Cambridgeshire; however it has been suggested that it was never made in the village (although there are some authorities who claim this is a myth). Stilton was a major trading post between London and Edinburgh, where the cheese was soon being sold as it was conveniently close to where the cheese was being produced (Hickman, 1996).

Until the late 20th Century, Stilton was made using unpasteurised cow’s milk; however between November 1988 and January 1989, 155 people in 36

outbreaks suffered from gastrointestinal symptoms due to the consumption of Stilton cheese, although the cause of the outbreak was unknown. This led to the alteration in Stilton production so that pasteurised milk had to be used (Maguire *et al.*, 1991).

There are currently only five licensed dairies within the three counties of Nottinghamshire, Derbyshire and Leicestershire (including Rutland) that can produce both Blue and White Stilton, although there is a sixth dairy that is licensed to produce White Stilton only. All six are represented by the Stilton Cheese Makers Association (SCMA) which was formed in 1936. Stilton cheese itself was only granted a product of designated origin (PDO) by the EU in 1996 (Ilbery and Kneafsey, 2000).

1.1.3 STILTON PRODUCTION

According to the Long Clawson Dairy (LCD), Stilton production begins by adding a starter culture to heated pasteurised milk. This starter contains lactic acid bacteria (often *Lactococcus lactis*) and *Penicillium roqueforti*. The lactic acid bacteria acidifies the milk whilst the growth of *P. roqueforti* mould promotes ripening of the cheese during the maturation stages. Rennet (a milk clotting agent) is also added to the milk to form curds.

Once the curds have formed, the whey is removed and the curd is left to drain overnight. The next day the curd is cut into six inch blocks before being milled, salted at a rate of 2.5-3% (w/v) and tipped into cylindrical cheese hoops (moulds). Each hoop stands on its own square draining board for four days, being turned daily to allow for equal drainage throughout the cheese. Stilton has to be made in a cylindrical shape due to the PDO guidelines and is an un-pressed cheese where the curds settle under their own weight.

After five days the hoops are removed and the surface of the cheese is smoothed over and wrapped in film to add support to the fragile curds and also to prevent exposure to the air, stopping the growth of aerobic microflora like *P. roqueforti*. The cheese is kept in a cool room for three days, where temperature and humidity are maintained (~10°C/85% humidity) and the cheese is turned daily. They are then moved to a maturation room where they continue to be turned daily for the next seven days to ensure even development of the curds.

After six weeks, the cheese is pierced with stainless steel needles to allow air to enter the centre of the cheese. This process is repeated in the seventh week. Piercing the cheese activates the *Penicillium roqueforti* encouraging mould sporulation to take place leading to the development of blue veining. After the second piercing, the cheese is left for seven days when their ripeness and the openness of the texture are graded. Those that do not reach Stilton standards (appearance, blue veining, aroma, etc) are sold as Blue Cheese (LCD, 2012).

1.1.4 MICROFLORA OF STILTON

1.1.4.1 Bacterial Flora of Stilton

When Stilton was made using unpasteurised milk, the microflora from the milk would often end up in the final Stilton product. A study by Yunita (2010) on Stichelton (a Stilton-like cheese made from raw unpasteurised milk) showed that bacteria found in the raw milk such as *Enterococcus faecalis* were present in the final Stichelton product (Yunita, 2010).

Now that Stilton is made with pasteurised milk, the pasteurisation process (70°C/20secs) removes any vegetative bacterial cells in the raw milk.

However, one study by Ercolini *et al.* (2003) showed that although Stilton is

made from pasteurised milk, a number of other bacteria were found in the final flora other than the starter. The authors used a number of different media for the viable counts and from that showed other bacteria present other than the *Lactococcus* used as the starter. For example growth on Rogosa agar showed the presence of *Lactobacillus*. Using 16S rDNA PCR DGGE analysis, they identified species of bacteria present such as *Lactobacillus plantarum* and a number of *Staphylococcus* species. The areas of the cheese colonised by these bacteria were demonstrated using fluorescence in situ hybridisation (FISH) which showed the presence of bacteria other than *Lactococcus lactis* in the blue veins (Ercolini *et al.*, 2003).

A further finding from the work by Yunita (2010) was that the lactic acid starter culture, *Lactococcus lactis*, can be found in the final Stichelton product. In contrast Ercolini *et al.* (2003) found that during the Stilton making process, the levels of *Lactococcus lactis* decreased as numbers of non-starter bacteria increased. These included species of *Lactobacillus* and *Leuconostoc* (Ercolini *et al.*, 2003).

Yunita also showed the presence of possible bacterial pathogen contamination through the identification of *Staphylococcus aureus* during the production of Stichelton. The contamination could have been by skin contact, or from machinery. By the final product however, *Staphylococcus* numbers had significantly decreased and no *S. aureus* were found, which is beneficial as *S. aureus* is a pathogenic bacterium and can cause illness if consumed. The dangers of having *S. aureus* present in cheese were highlighted by two outbreaks of food poisoning in Brazil where 50 individuals were taken ill

during the first outbreak and 328 individuals during the second where the source of the outbreak was Minas cheese (Do Carmo *et al.*, 2004).

1.1.4.2 Fungal Flora of Stilton

Yeasts are often found growing during the maturation (ripening/curing) stage of some cheese varieties. These yeasts are often known as secondary flora of the cheese as they are nearly always contaminants of the cheese and are rarely added as a starter culture. Contamination by yeast often comes from process equipment, salt and any localised fungal cultures (Fleet, 1990). There are also cases of yeast contamination of the rennet (Martinez *et al.*, 1986).

Fungi are also used to encourage the growth of other microorganisms in surface-ripened cheeses. An example of this is *Penicillium camemberti* which gives Brie and Camembert the white mycelial growth on the surface of the cheese (Fox *et al.*, 2004).

In Stilton, *P. roqueforti* spores are added as a starter culture which gives the final product its typical blue cheese smell and taste. The lack of air reaching the cheese surface by wrapping, as in Stilton production, reduces the ability of *P. roqueforti* to grow as it is aerobic. During the piercing stage of Stilton production, air penetrates the cheese allowing the mould to grow, promoting sporulation and giving the blue veins typical of Stilton. Over the maturation period leading to piercing, the cheese develops a coat of yeasts which inhibit and restrict the growth of *P. roqueforti* so that the mould does not engulf the cheese. It is thought that these yeasts that contaminate the surface of the cheese are found in the maturing rooms of the dairies (Welthagen and Viljoen, 1998).

Work by Gkatzionis (2010) showed that the yeasts found in Stilton were species of *Kluyveromyces lactis*, *Trichosporon beigleii*, *Debaryomyces hansenii* and *Yarrowia lipolytica*. He showed that these yeast species were found on the surface, in the veins and in the white core of the cheese, just under the surface (Gkatzionis, 2010). This was developed further by Wei (2011) who showed each species contributes different flavours to the Stilton, and work in conjunction with each other and the *P. roqueforti* to give Stilton its unique taste (Wei, 2011).

1.2 FLAVOUR ANALYSIS

1.2.1 STILTON AROMA

The aroma of Stilton varies between cheeses. It is mainly affected by the starter microorganisms (*Lactococcus lactis* and *P. roqueforti*) and the secondary flora (different yeast species and non-starter bacteria). There are other factors that may affect the aroma including enzymes from the rennet, and sometimes enzymes in the milk, although these are normally reduced during pasteurisation (Urbach, 1997).

The *P. roqueforti* ripens the cheese during maturation, and both its conidia and mycelia are capable of producing aroma compounds (Fan *et al.*, 1976). It is responsible for a major part of the lipolytic and proteolytic activity and is involved in the metabolism of fatty acids and amino acids, giving a number of ketone and alcohol compounds (Kinsella and Hwang, 1976). It is the ketones produced that are responsible for the typical blue cheese smell.

The yeasts that contaminate Stilton are thought to produce aroma compounds (Martin *et al.*, 2001). A study by Gkatzionis *et al.* has shown that different

Stiltons produce different aroma compounds, including a variety of ketones, acids, esters and alcohols (Gkatzionis *et al.*, 2009). These authors have also shown that different parts of the cheese produce varying aroma compounds. The ketones dominated the flavour volatiles and the highest concentration of these volatiles could be found in the blue veins where they are likely to have been produced by *P. roqueforti*. More alcohols were contained in the white core of the cheese compared to the outer crust and blue veins. The authors concluded that the different sections of the cheese contained different flavour volatiles, and that the blue veins and outer crust contained the most ketones whilst the white core contained more alcohols and aldehydes (Gkatzionis *et al.*, 2009).

1.2.2 SOLID PHASE MICROEXTRACTION GAS CHROMATOGRAPHY-MASS SPECTROMETRY (SPME GC-MS)

Gas Chromatography (GC) has a high resolution and can detect trace components in complex mixtures. This means that when used with Mass Spectrometry (MS) it becomes a useful technique in the analysis of food (Careri *et al.*, 2002).

Using headspace sampling allows for the extraction of volatiles produced by a given food substance. Solid Phase Microextraction (SPME) is a technique from which volatile compounds can be analysed. As it does not involve the use of any solvents, the amount of contamination by the machine is greatly reduced (Careri *et al.*, 2002).

An inert fibre is used to absorb the volatiles in the headspace, and these are then desorbed into a GC column. Volatiles present in the headspace can then be detected and quickly analysed (compared to standard chromatography). A

previous study has used this technique in experiments involving Camembert cheese and has shown that headspace SPME GC-MS is one of the better techniques used to analyse aroma volatiles (Jaillais *et al.*, 1999).

2 MATERIALS AND METHODS

2.1 MEDIA AND CHEMICALS

Unless specified, all media and chemicals used were obtained from Oxoid Ltd, Hampshire, UK. The agar used was Brain Heart Infusion Agar (BHI), M17 Agar, MRS Agar, Rose Bengal Chloramphenicol Agar (RBC) and Plate Count Agar (PCA), and the broth used was MRS broth. The chemicals used were Phosphate Buffered Saline (PBS), Maximum Recovery Diluent (MRD) and ¼ Strength Ringers Solution. All media and chemicals were prepared using the manufacturer's instructions (Appendix 1). After pouring, the agar was stored at 4°C as was the broth. The chemicals were all stored at room temperature.

2.2 STRAINS AND CULTURE

The *Lactococcus lactis* subsp. *lactis* used was a strain from the University of Nottingham culture collection.

The *Penicillium roqueforti* strain used was strain SO17 and was isolated from a mature Stilton cheese. This was provided by Dr Konstantinos Gkatzionis (University of Northampton).

The yeast strains used were *Kluyveromyces lactis* SB15, *Debaryomyces hansenii* Y12 and *Trichosporon beigleii* Y18. These were isolated from mature Stilton cheeses. They were provided by Dr Konstantinos Gkatzionis (University of Northampton).

The strains of yeasts, *Penicillium roqueforti* and *Lactococcus lactis* were stored at -80°C in 20% (w/v) glycerol solution.

For growth of the bacterium a glycerol bead of *Lactococcus lactis* was added to 10mL MRS broth and incubated at 30°C for 24h. Using the streak

technique, a loopful of the MRS broth was streaked on BHI Agar and incubated at 30°C for 24h, before being stored at 4°C until use.

For culture of fungi from the glycerol solutions, 10µl of *Penicillium roqueforti* and the yeast strains were spread onto separate RBC Agar plates using the spread technique and incubated at 25°C for 5 days before being stored at 4°C until use.

2.3 MILK MODEL FOR VOLATILE PRODUCTION

2.3.1 PREPARATION OF THE *LACTOCOCCUS* INOCULANT

Using a sterile wire loop, a colony of *Lactococcus lactis* on BHI agar was put into 10mL of MRS broth and was incubated at 30°C for 24h. From this 1mL of culture was centrifuged for 2min at 9000g using a Hettich Mikro 200 Centrifuge (DJB Labcare Ltd, Buckinghamshire, UK). The supernatant was removed and the pellet was washed with 1mL PBS. The PBS was removed and the pellet was resuspended in 1mL of fresh PBS giving ~10⁹cfu/mL. A serial dilution was prepared using MRD to give ~10⁶cfu/mL of *Lactococcus lactis* to give a stock culture.

2.3.2 PREPARATION OF THE *PENICILLIUM* INOCULANT

With a cotton bud, conidia of *P. roqueforti* were swabbed off RBC agar and suspended in 10mL of PBS. Using a haemocytometer, the number of conidia per mL was counted. A stock of ~10⁷conidia/mL was required, so more conidia were added if needed, and further dilution was carried out if there were too many.

2.3.3 PREPARATION OF THE YEAST INOCULANT

Using a cotton bud, yeast cells were swabbed off RBC agar and suspended in 10mL of PBS. Using a haemocytometer, the number of cells per mL was counted. A stock of $\sim 10^7$ cells/mL was required, so more cells were added if needed, and a dilution was carried out if there were too many.

2.3.4 INOCULATION AND INCUBATION OF THE MILK MODEL

The milk used was Sainsbury's UHT whole milk standardised to less than 4% fat which came in 1L cartons. Before opening, the top of the milk carton was cleaned using 100% ethanol. 100mL of milk was poured directly into a sterile 250mL Duran bottle containing a magnetic stirring flea. Inoculation cultures (1mL of each as appropriate) were added to each Duran bottle. The Duran bottles were incubated statically at 25°C for 10 days. Using magnetic stirrers, the milk solutions were stirred for 5min every other day during incubation. After 10 days, samples were dispensed into 20mL vials ready for SPME GC-MS analysis.

2.4 CHEESE PRODUCTION MATERIALS

The milk used for cheese production was Sainsbury's UHT whole milk standardised to less than 4% fat which came in 1L cartons. For salting, Tesco British Cooking Salt was used.

The commercial starter culture that was used was OV100 which was a freeze dried preparation and was obtained from Orchard Valley Dairy Supplies, Worcestershire, UK. It contained *Lactococcus lactis*, *Lactococcus cremoris* and *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis*. The starter culture was stored at 4°C. The *Penicillium roqueforti* used was *P. roqueforti* PRB6

liquid culture and was also obtained from Orchard Valley Dairy Supplies, and was stored at -20°C. The rennet used was Animal Rennet GM Free obtained from Moorlands Cheesemakers Ltd., Somerset, UK.

For cheese production, the materials used were two 12L aluminium buckets with lids, an aluminium draining spoon, an aluminium baking tray, three 80mm diameter by 90mm height plastic cylindrical cheese moulds with solid bases and plastic followers and muslin cheese cloth obtained from Moorlands Cheesemakers Ltd. All materials were autoclaved before use.

To create certain humidity levels around the cheese during incubation, cheeses were stored in containers with excess salt solutions. The different salt solutions and the humidity levels they produce are given in Appendix 2. All salts were obtained from Fisher Scientific, Leicestershire, UK.

2.5 CHEESE SAMPLES

A number of commercial cheese samples were used for testing. The commercial cheeses used were Sainsbury's 200g Wensleydale, Sainsbury's 200g So Organic Stilton (made by Cropwell Bishop), Sainsbury's 200g Shropshire Blue and Castello 150g Danish Blue Cheese. These were compared to cheese samples made using the cheese production method described in Section 3.1.

2.6 SOLID PHASE MICROEXTRACTION GAS

CHROMATOGRAPHY-MASS SPECTROMETRY (SPME GC-MS)

GC-MS settings were: The temperature of the injection port was 220°C.

Chromatography was carried out using a ZEBRON Phase: ZB5 gas

chromatography column (30m x 0.53mm x 1.50 μ m) from Phenomenex (Cheshire, UK) in a Trace GC Ultra 2000 mass spectrometer (Thermo Fisher Scientific Inc., Massachusetts, US). Helium was employed as the carrier gas, at a constant pressure of 17 psi. A 10mm Stableflex fibre coated with 50/30 μ m divinylbenzene-carboxen on polydimethylsiloxane bonded to a flexible fused silica core (Supelco, Pennsylvania, US) was used for a syringe fibre method for splitless autosampling.

The oven temperature programme was as follows: an initial temperature of 40°C was maintained for 2min, increasing at a rate of 8°C/min to a final temperature of 220°C. The transfer line from the gas chromatograph to the mass spectrometer was held at 250°C. The mass spectrometer was operating in positive ionisation electron impact mode (EI+) at 70 eV. The detector was operated in scan mode (2scans/s), scanning from m/z 20 to 250. Source temperature was 200°C.

Xcalibur 1.4 SR1 program (Thermo Fisher Scientific Inc.) was used to process all data and set up all settings. The compounds were identified by comparing their retention times and mass spectra with retention indices (RI) and mass spectra published in databases of the National Institute of Standards and Technology (NIST) mass spectral library.

The Xcalibur programs: Qual Browser and Quan Browser were used for analysis alongside SPSS Statistics 19 (IBM, New York, US) and The Unscrambler v9.0 (Camo Process AS., Oslo, Norway).

The sampling method used was the same for both the milk and cheese samples. For all the samples, 4 replicates were prepared with 2 extra vials per run used to clean the machine of any compounds left from previous testing

prior to the tests. Samples (5mL) from the milk model system were dispensed into 20mL Headspace vials (22.5mm by 75.5mm; Grace Alltech, Lancashire, UK) and immediately sealed with a magnetic cap (20mm diameter, 5mm centre, PTFE/Silicone Liner; Grace Alltech). For cheese samples, 2g of each sample was put into 20mL Headspace vials and immediately sealed with a magnetic cap and allowed to equilibrate at 22°C for 30min prior to analysis.

2.7 VIABLE COUNT

The Miles and Misra technique (Miles *et al.*, 1938) was used for the viable counts. Samples for testing were taken during the cheese making process. The media used were M17 for Lactococci, MRS for Lactic Acid bacteria and PCA for total aerobic count. RBC was used to enumerate yeasts and moulds.

For the starter culture sample, 0.1g of starter culture was added to 9.9mL of MRD to give a dilution of 10^{-2} . For the milk (1mL) and cheese (1g) samples 9mL of MRD was added to give a dilution of 10^{-1} . Serial dilutions were carried out using MRD as the diluent up to 10^{-11} for the starter culture sample and 10^{-10} for the cheese and milk samples.

For yeasts and moulds 100µL of the initial dilutions was spread onto RBC and incubated at 25°C for 5 days. For bacterial counts three 20µL spots of each dilution were spotted onto M17, MRS and PCA agar plates and were incubated at 30°C. The M17 and MRS plates were incubated for 24h whereas the PCA plates were incubated for 48h. The number of colonies in each spot was counted, and the spots containing between 3 and 30 colonies were used to work out the viable count of the samples using the following calculation:

Number of cells per g = Number of cells x 50 x 1/Dilution Factor

2.8 pH TESTING

The pH was tested during the cheese production method using Whatman pH 3.8-5.5 and 5.2-6.8 indicator papers (Whatman International Ltd., Kent, UK).

2.9 TEXTURE ANALYSIS

The texture of cheese samples was measured using the TA.HD Plus Texture Analyzer (Texture Technologies, New York, US). The cheese made using the production method was compared to commercial cheeses (Section 2.5) with 1cm³ used from each sample; this test was repeated 3 times.

Texture Exponent 32 (Visual Components Inc., Surrey, UK) was the program used to set up the equipment and was used to analyse the data. A 2-bite test method was set up with a 10mm diameter cylinder probe. The pre-test and the post-test speed was 5mm/sec with a test speed of 1mm/sec. The probe was set up to press 10mm into the cheese and the test was triggered at a force of 5g. The data were analysed by Texture Exponent 32.

2.10 WATER ACTIVITY

The water activity (a_w) of the cheese made using the production method was compared to the commercial cheeses (Section 2.5). A sample of cheese was placed into a plastic cup in the drawer of the Aqua Lab Series 3TE (Decagon Devices Inc., Washington, US) and left until the machine signalled the a_w of the sample on the digital display. This method was replicated twice for each cheese sample.

3 RESULTS

3.1 CHEESE PRODUCTION METHOD

3.1.1 INITIAL CHEESE PROTOCOL

A cheese production method was provided by Joe Schneider of Stichelton Dairy Ltd, Nottinghamshire, UK. Stichelton production is similar to that of Stilton; however it uses unpasteurised milk rather than pasteurised milk. The method in Figure 1 was developed for the production of a small-scale Stilton cheese model.

The cheese model production procedure was designed to give cheeses of approximately 400g. Because it was to be used to examine added flora effects, no background flora was desired; therefore the milk chosen was a UHT whole milk (Section 2.4). Initially ten litres of milk was chosen as a manageable working volume providing 3 replicate cheeses per batch based on a 10% curd volume production. Although Stilton is an un-pressed cheese, the small size of the model cheese to be made meant the use of a follower was required in order to help the curd settle.

When the Stichelton protocol was applied for the first time, it was made with 10L of milk to which 0.0002g of starter culture, 0.24mL *P. roqueforti* spores and 1.5mL of rennet was added. The amount of *P. roqueforti* spores used was that recommended by the supplier (Orchard Valley Dairy Supplies) who suggest 1 dose (12mL) should be used per 500L milk.

The result of this attempt was that after adding the rennet and incubating for 90min the curd and whey did not separate out, leaving a cream-like product. Separation did not occur even when incubated for 24h for curdling.

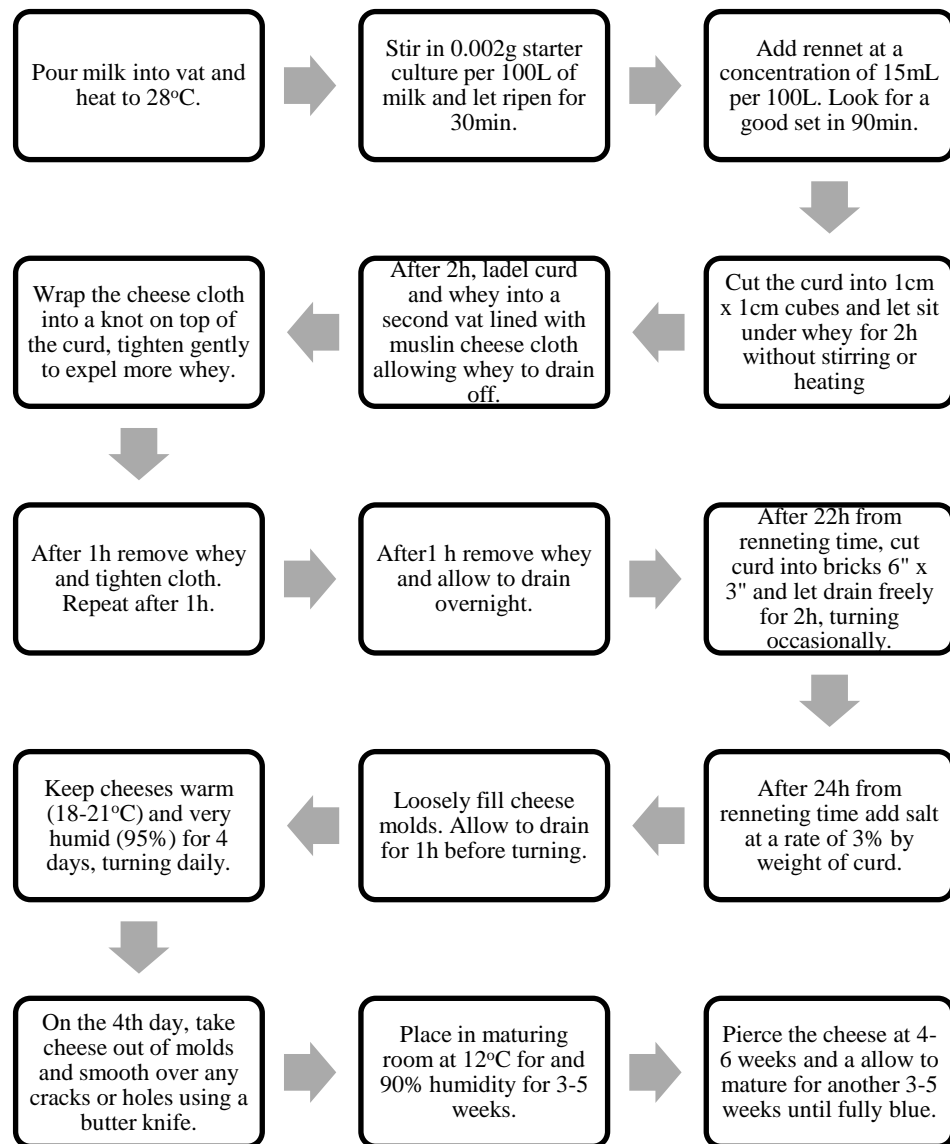


Figure 1: The blue cheese production protocol as it was provided by Joe Schneider, Stichelton, Nottinghamshire, UK.

3.1.2 IMPACT OF STARTER CULTURE

The failure of the milk to ferment may have been due to the level of starter culture used. The weight used was based on making a raw milk cheese and it is likely the natural flora in the milk contributed to the fermentation. For a sterile milk cheese more organisms would be needed. Work on other cheeses suggested that a higher volume of starter culture would be required to separate out the curd and the whey. One study showed that a rate of 1.5% (w/v) of commercial starter culture was required in the production of a raw milk cheese (Kameni *et al.*, 2006) where as in another study a high yield of cheese was produced when using a rate of 2% (w/v) (Najafi *et al.*, 2008). The starter culture was subsequently increased to 2% (w/v) compared to the 0.002% (w/v) that was used in the initial protocol using a weight of 0.2g/L.

An initial test was carried out to see how the increase in starter culture would affect the pH of the milk, and how long an incubation was needed after the addition of the starter culture for the milk to reach the required acidity of pH4-5. Two Duran bottles each containing 100mL milk had 2% (w/v) of starter culture added to them and were incubated at 28°C for 24h. The pH was tested after 4, 8 and 24h using pH3.8-5.5 and 5.2-6.8 indicator papers (Whatman International Ltd.). Figure 2 shows that over a 24h period, the starter culture acidified the milk to pH4.8. This meant that for the next attempt, the milk would be incubated for 24h after the addition of the starter culture to allow for curdling.

For the second cheese making attempt, 5L of milk was used and was inoculated with 0.1g of starter culture together with 0.12mL of *P. roqueforti*.

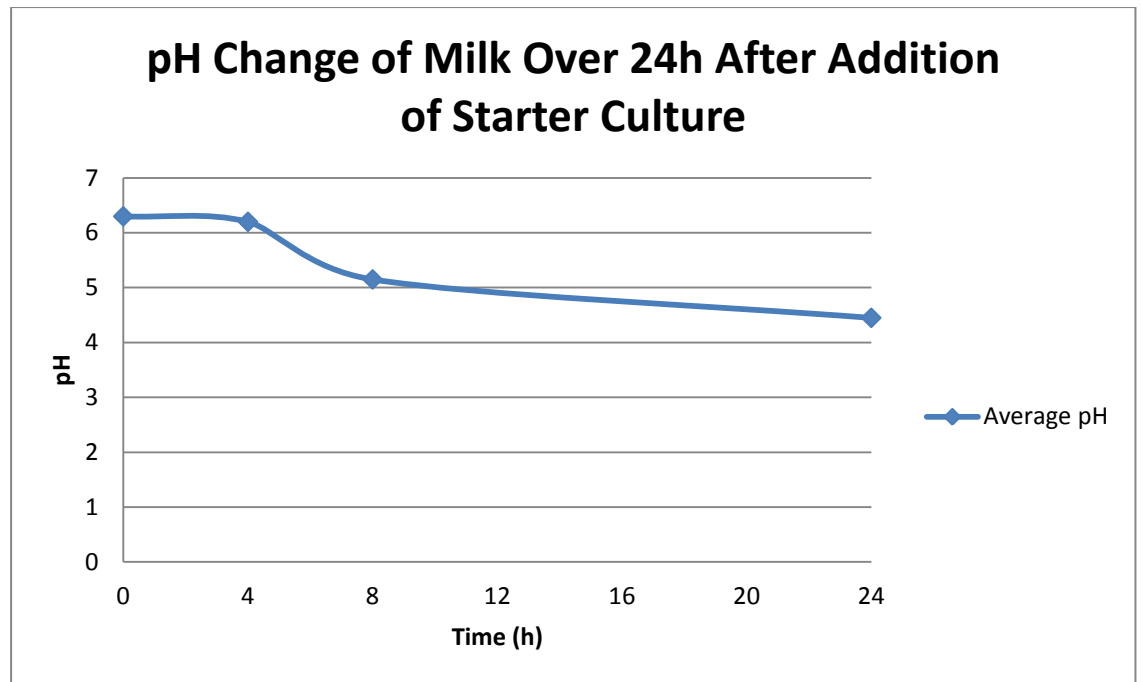


Figure 2: The average change in pH of two replicates incubated at 28°C over 24h of 100mL of milk with 2% (w/v) of starter culture added.

In addition to this, 1.25mL of rennet was used as the supplier (Moorlands Cheese Makers Ltd.) suggested using 1mL per 4L of milk.

After being left to curdle for 24h, the curd was cut up and left to sit under the whey for 2h without stirring. The result of this was that the curd and whey mixed together forming a yoghurt-like texture, from which no curd could be separated.

3.1.3 ALTERING THE STICHELTON PROTOCOL FOR A SMALL-SCALE MODEL

Using 5L of milk and the same volumes of inoculants and rennet used in Section 3.1.2, a third cheese making attempt was carried out, but instead of cutting the curd, it was scooped from the aluminium bucket into the muslin cloth. This made the separation of the curd and whey possible.

After the curd had been left overnight for the whey to run off, a ~1200g brick of curd was left for another 2h to drain (this was not cut into smaller bricks as the mass was already small enough for drainage and salting). To the brick, 36g of salt was mixed in by hand (wearing gloves) before 400g was put into each of three plastic cheese moulds with a follower placed on top of each and left to drain for 1h, before being turned and left to drain for another hour. These were incubated at 20°C for 4 days at ~97% humidity (Section 2.4).

After being incubated, the cheese was removed from the moulds, and using a sterile disposable spreader, the surface was smoothed, filling over any cracks or holes in the surface of the cheese. The cheese was then incubated at 10°C with a barium chloride solution used to maintain ~90% humidity. The barium chloride dissolves into the water so the water diffuses into the air due to osmosis.

Within a week of being out of the moulds, the *P. roqueforti* had grown uncontrollably, engulfing the cheese as shown in Figure 3. This prevented any further maturing or testing of these cheeses.

While the cheese was incubating at 97% humidity it was noted that the cheese had a very soft texture. The high humidity used in commercial cheese making stops the cheese from drying out whilst maintaining a solid texture. Within these small-scale cheeses, it was apparent that they had drawn up water, and had softened too much. This may have contributed to the overgrowth of the mould. From this it was deduced that a lower humidity would be required for further attempts.

3.1.4 REMOVAL OF *P. ROQUEFORTI*

The method from Section 3.1.3 was repeated without the addition of *P. roqueforti* to see if a cheese with a better texture could be produced initially. A lower humidity of ~75% was also used for all cheese incubations.

From this, a white cheese was produced; however it showed signs of *P. roqueforti* surface growth which shows the ease with which *P. roqueforti* can contaminate the cheese through the environment (Figure 4).

After 1 week of maturing, the texture of the cheese was tested using the method in Section 2.9 and was compared to a number of commercial cheeses (Section 2.5). Figure 5 shows the differences between the cheeses. The first peak shows the hardness of the cheese, and the area between force equals zero and the tip of the second peak shows the springiness of the cheese.

Wensleydale was much harder than the other commercial cheese which is expected as it is a pressed cheese. The Stilton and Shropshire Blue have a



Figure 3: Two of the cheeses having been engulfed by *P. roqueforti* preventing any further maturing and testing.

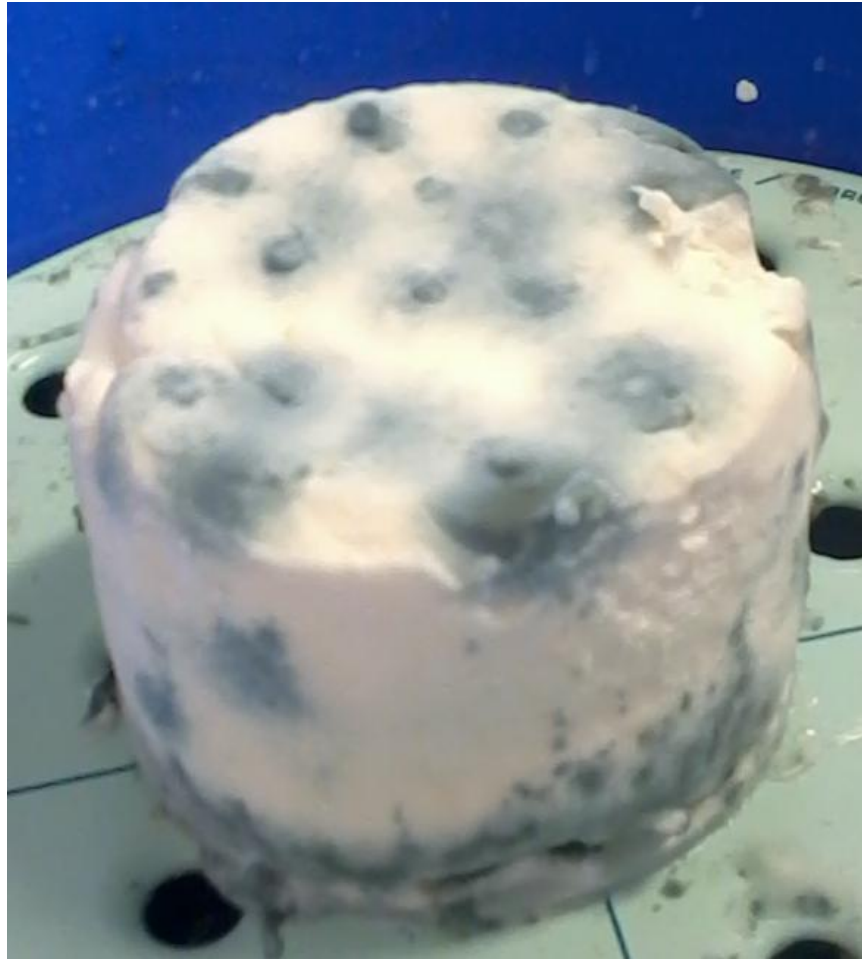


Figure 4: A white cheese with *P. roqueforti* contamination one week after coming out of the mould.

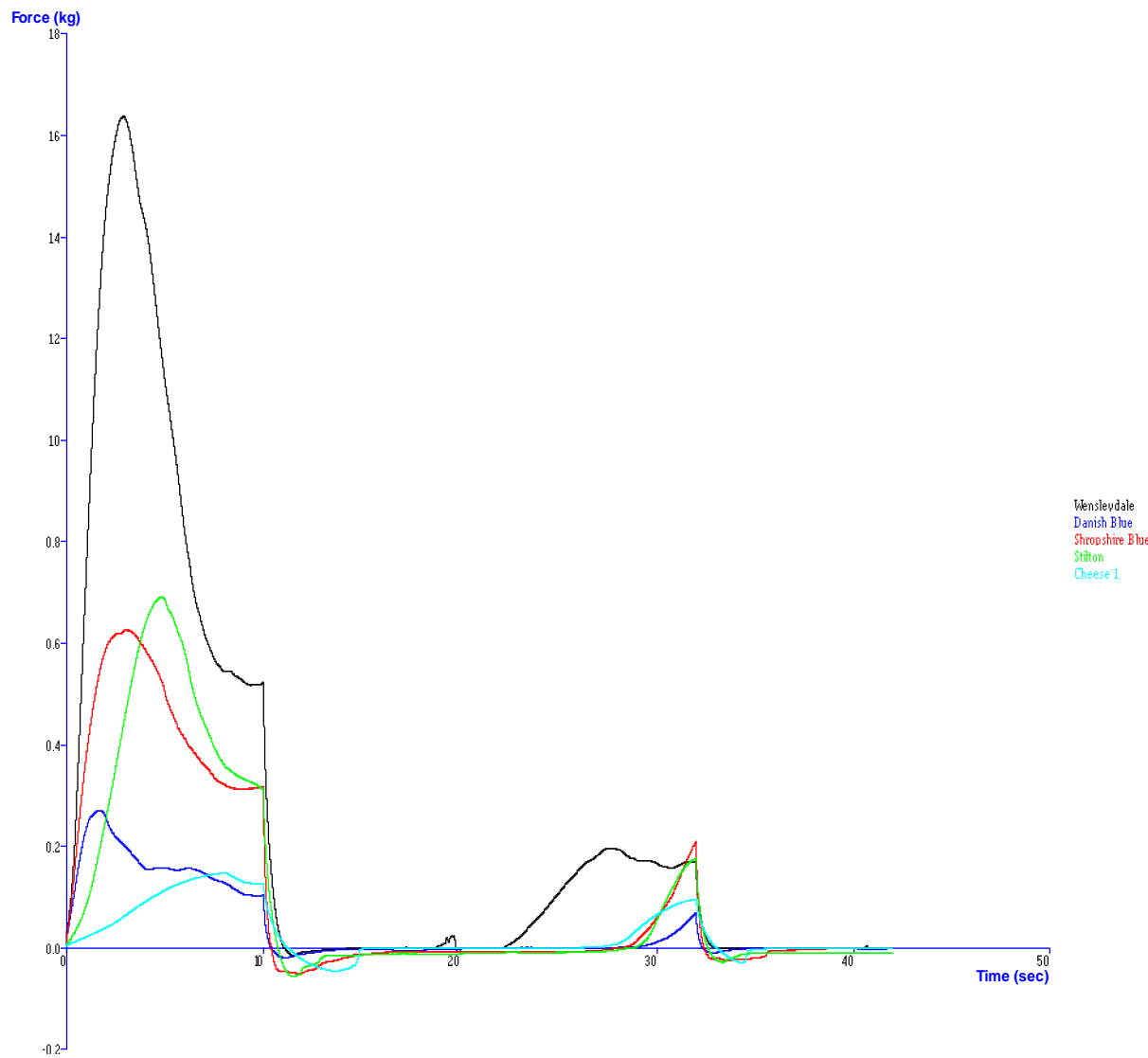


Figure 5: The texture of 4 commercial cheeses compared to the first small-scale cheese. Black = Wensleydale; Blue = Danish Blue; Red = Shropshire Blue; Green = Stilton; Light Blue = Sample 1.

similar hardness as they are made by the same process; however Shropshire Blue contains a natural food colouring called Annatto to give the cheese its orange colour. Danish Blue is softer than Stilton, although made in a very similar way. This is because during maturing, Stilton is turned frequently to allow for more drainage of moisture; however Danish Blue is not turned, and therefore retains more moisture, making it a softer cheese.

Figure 5 shows that the peak of the initial hardness of the cheese sample is just over half the hardness of the Danish Blue, and not close to that of the commercial Stilton. As Danish Blue is softer than Stilton because it retains more water, the water activity of the cheese was tested to see if it was the cause of the softness of the cheese. Figure 6 shows the water activity of the commercial cheeses and the cheese sample. It shows that although the cheese sample is softer, it has a similar water activity to the Stilton and Shropshire Blue cheese and is much lower than Danish Blue.

The sample cheese could have been softer than the commercial cheese as during incubation in the molds, the curd was not being compressed as much. Although commercial Stilton is not a pressed cheese, when made using 11kg of curd, it gets compressed under its own weight. With this information, the weight of curd that would normally sit on the curd produced in this small-scale process was worked out, and weights were added to the top of the cheese. It was calculated that within a large-scale commercial cheese mould, 770g more curd would sit on top of the 400g of curd (with a diameter of 8cm and a height of 9cm) that would be placed in the moulds used in this project. In the commercial process when 11kg of curd is used in molds that have a diameter of 25cm and a height of 30cm. However, as commercial cheese is turned daily,

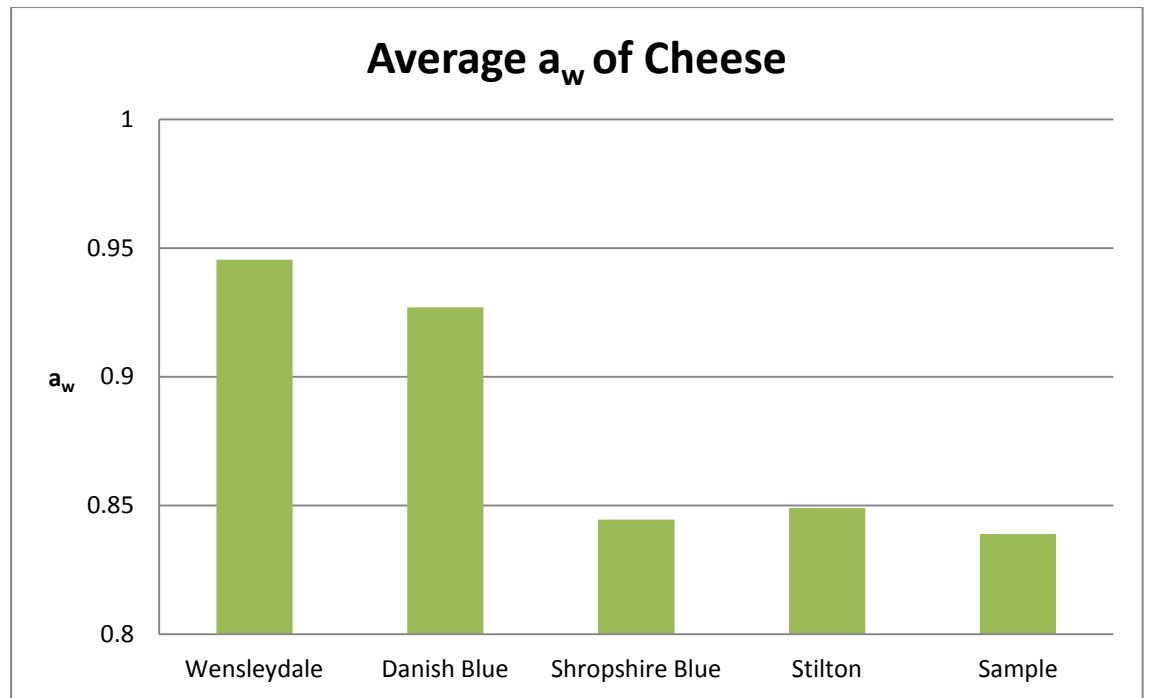


Figure 6: The average water activity of the commercial cheese compared to the first small-scale cheese.

arguably the curd only has weight on top for two days. This means if 385g of cheese were to be placed on top of the curd for 4 days, and the curd was turned

daily, this would simulate the weight that would press down on the curd during the commercial process whilst pressing all of the curd equally.

3.1.5 EFFECT OF PRESSING THE CHEESE

Three 400g cheeses were made using the same process from Section 3.1.4; however during this process, different weights were placed on top of each cheese during the first incubation stage to see if this had an effect on the texture of the cheese. The cheeses were then left for a week to mature. Figure 7 shows the differences between the texture of the three cheeses and the effect that the different weights had on them.

Figure 7 shows that the addition of the weight led to a harder cheese; however there is not much difference between the hardness of the cheeses with 350g and 500g weights on. This would suggest that although adding a weight to press the cheeses gives a harder texture, continually increasing the weight above a certain point does not continue to make the cheese harder.

To ensure that it was the pressing that was causing the increase in hardness, and not an increase in loss of water, the water activity was measured. The results can be seen in Figure 8 which shows that the water activity was similar to the un-pressed cheese and the commercial Stilton. This means that the added weight helped increase the hardness of the cheese without altering water activity so for further production, 350g weights would be placed on top of the cheese during initial incubation.

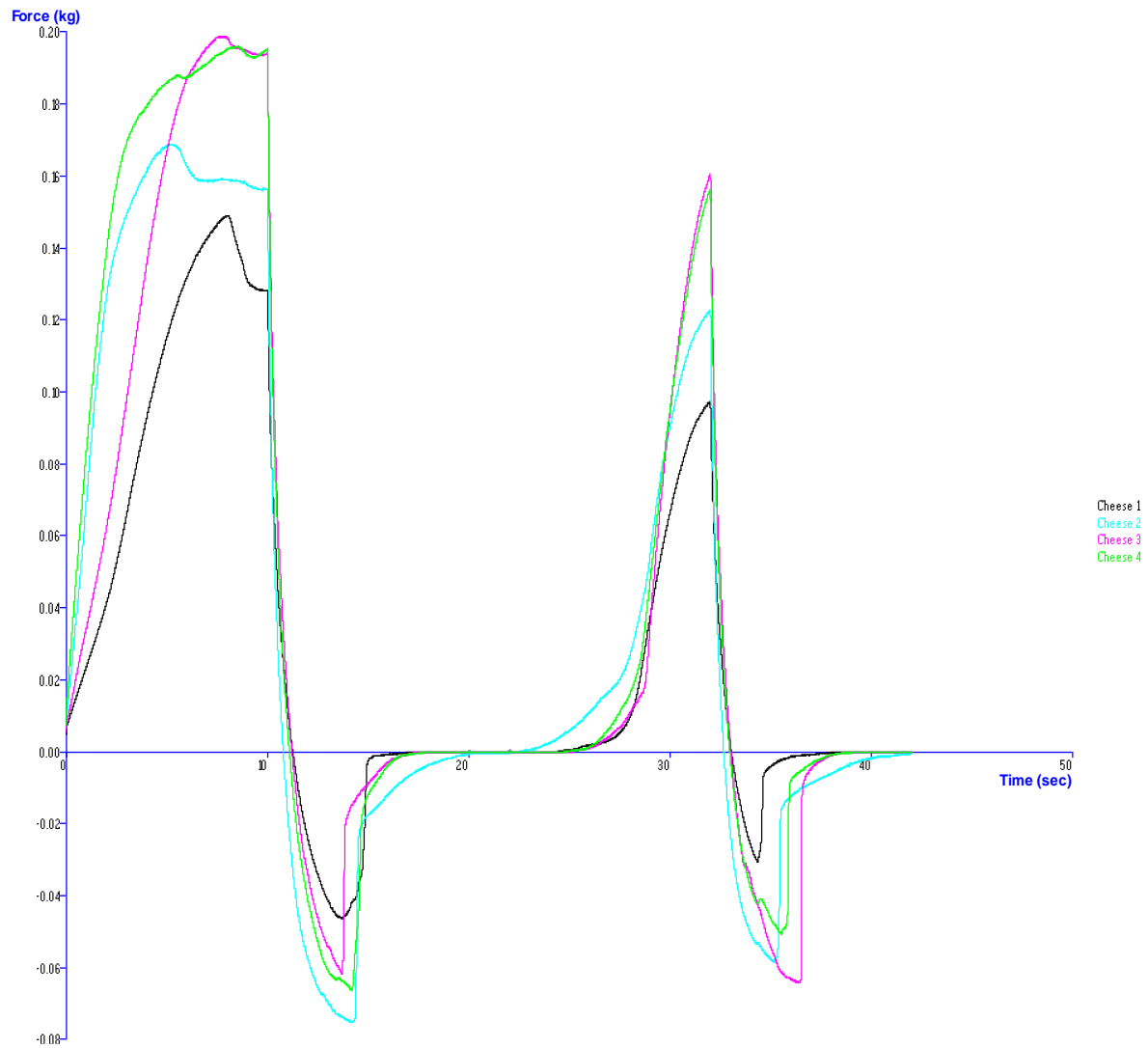


Figure 7: The texture of the 3 pressed cheeses compared to the un-pressed cheese from Section 3.1.4. Black = Un-pressed sample 1; Light Blue = Sample 2, pressed under 200g of weight; Pink = Sample 3, pressed under 350g of weight; Green = Sample 4, pressed under 500g of weight.

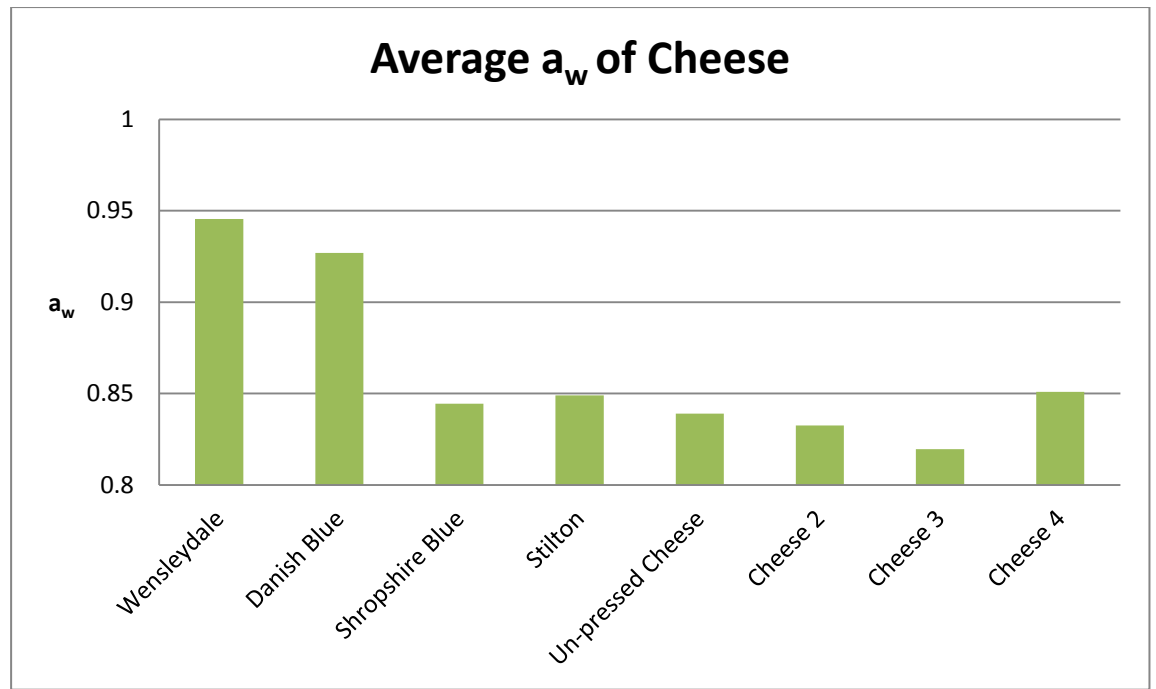


Figure 8: The average water activity of the commercial cheese compared to the sample cheese.

A comparison of these pressed cheeses with commercial cheeses is shown in Figure 9. Although harder than the previous un-pressed cheese, they were still a little softer than the Danish Blue and much softer than the Stilton.

3.1.6 EFFECT OF MATURATION

Using the same process as Section 3.1.5, three 400g cheeses were made. The texture of two of the cheeses was measured over 3 weeks. With the 3rd cheese, the Miles & Misra technique was used to determine the viable count of Lactic acid bacteria from the starter culture until three weeks into maturation.

Figure 10 shows the texture of the cheese samples over three weeks. Both cheeses increased in hardness from 1 week of maturation until 3 weeks of maturation. Figure 11 shows that the water activity was similar to that of Stilton throughout the maturation process.

After 3 weeks of maturation, the two cheeses had the same hardness as Danish Blue. Although not as hard as Stilton, they were harder than previous small-scale cheeses and considered appropriate for the model (Figure 12).

The viable counts of the cheese were calculated throughout the process to see the changes in levels of bacteria, and whether there was any evidence of fungal or bacterial contamination. The results of these can be seen in Appendix 3.

Figure 13 shows that the number of microflora during the cheese production process decreased during the salting of the curd. This is because the salt kills most of the lactic acid bacteria. The microflora begins to recover after salting but plateaus 11 days after salting, roughly 14 days into the process.

The number of bacteria on the M17 and MRS plates remains equal during the

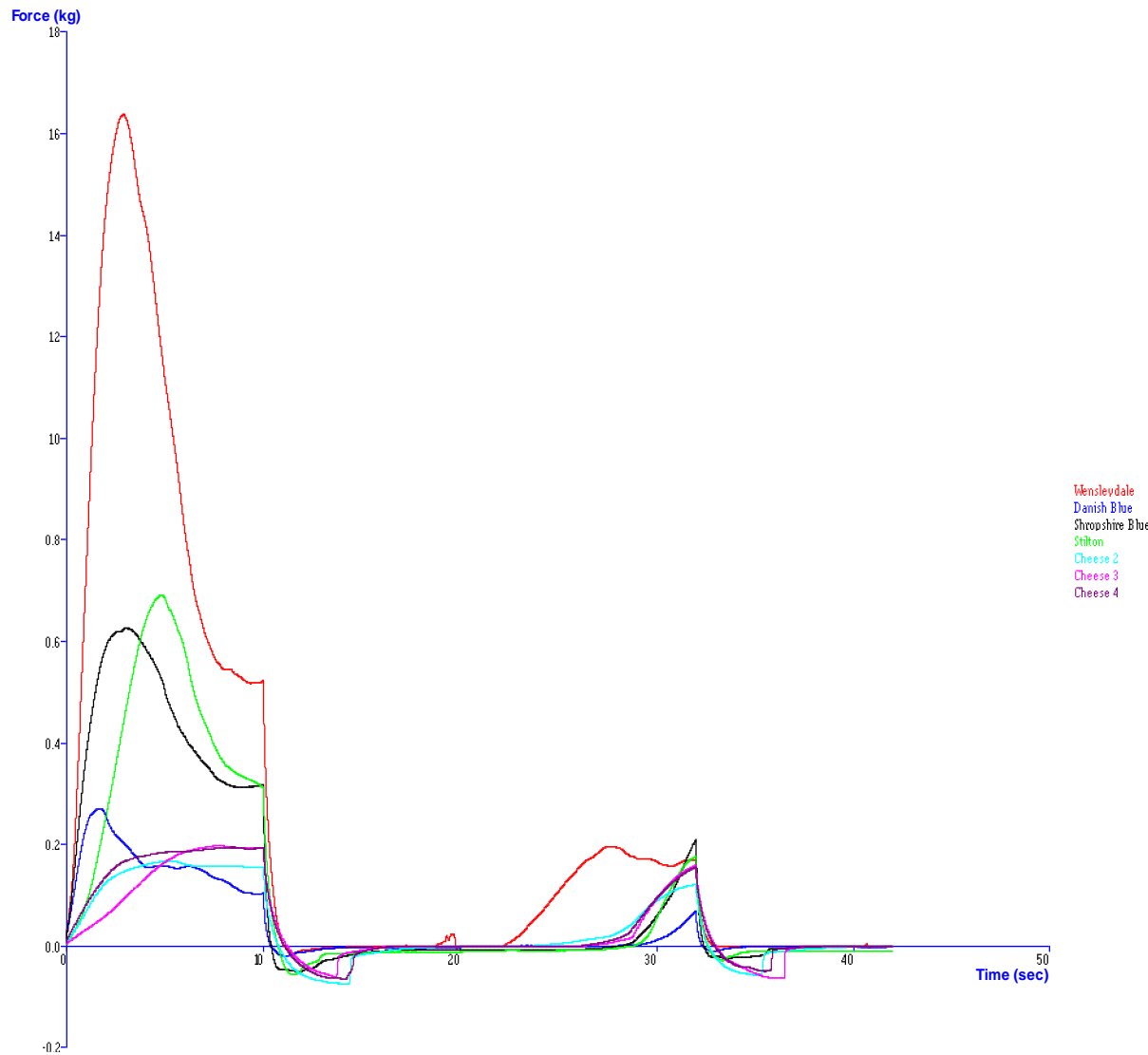


Figure 9: The texture of the 3 pressed cheeses compared to the commercial cheeses. Red = Wensleydale; Blue = Danish Blue; Black = Shropshire Blue; Green = Stilton; Light Blue = Sample 2, pressed under 200g of weight; Pink = Sample 3, pressed under 350g of weight; Purple = Sample 4, pressed under 500g of weight.

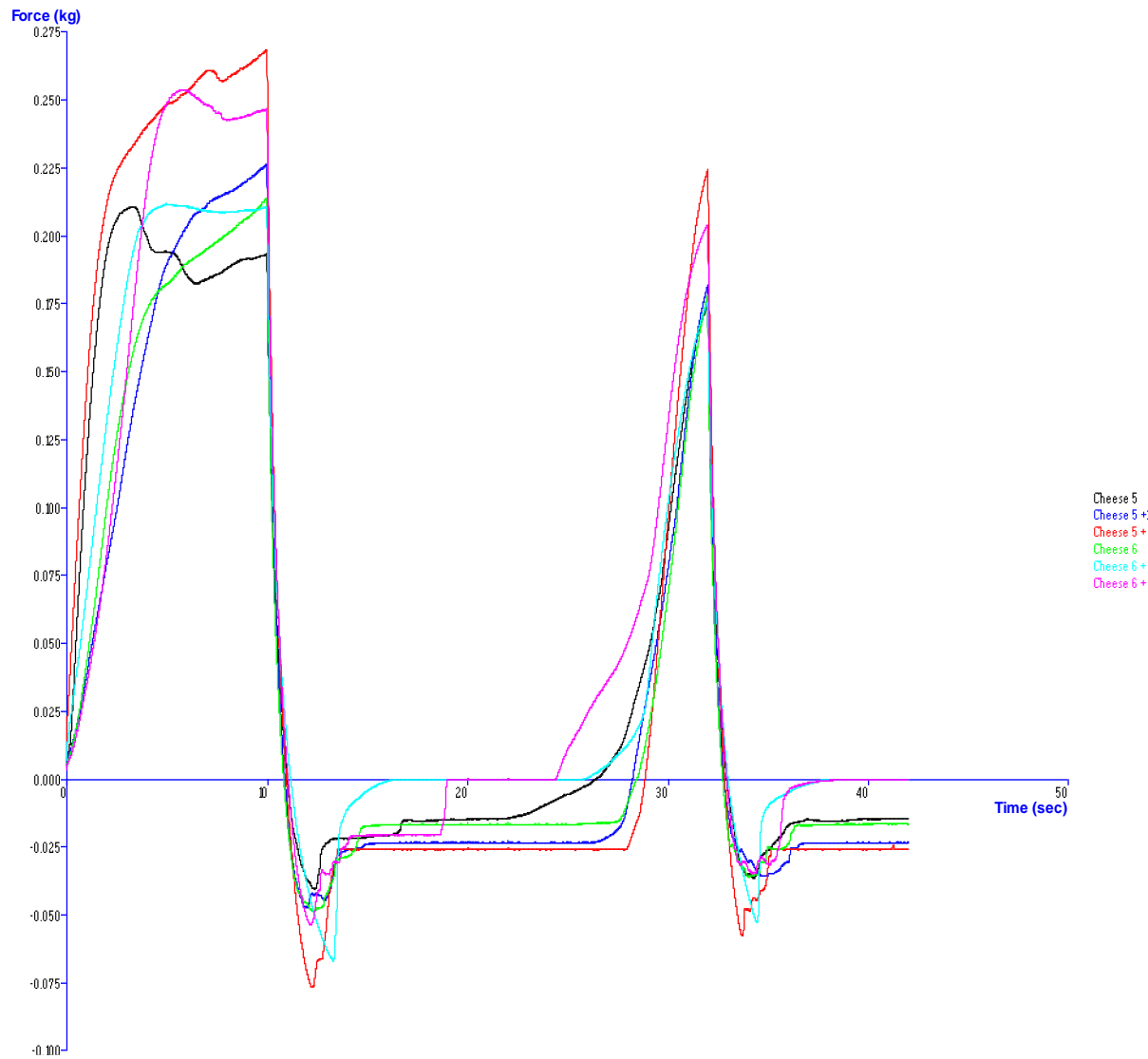


Figure 10: The texture of the two cheeses after 1 week, 2 weeks and 3 weeks of maturation. Black = Sample 5 after 1 week; Blue = Sample 5 after 2 weeks; Red = Sample 5 after 3 weeks; Green = Sample 6 after 1 week; Light Blue = Sample 6 after 2 weeks; Pink = Sample 6 after 3 weeks.

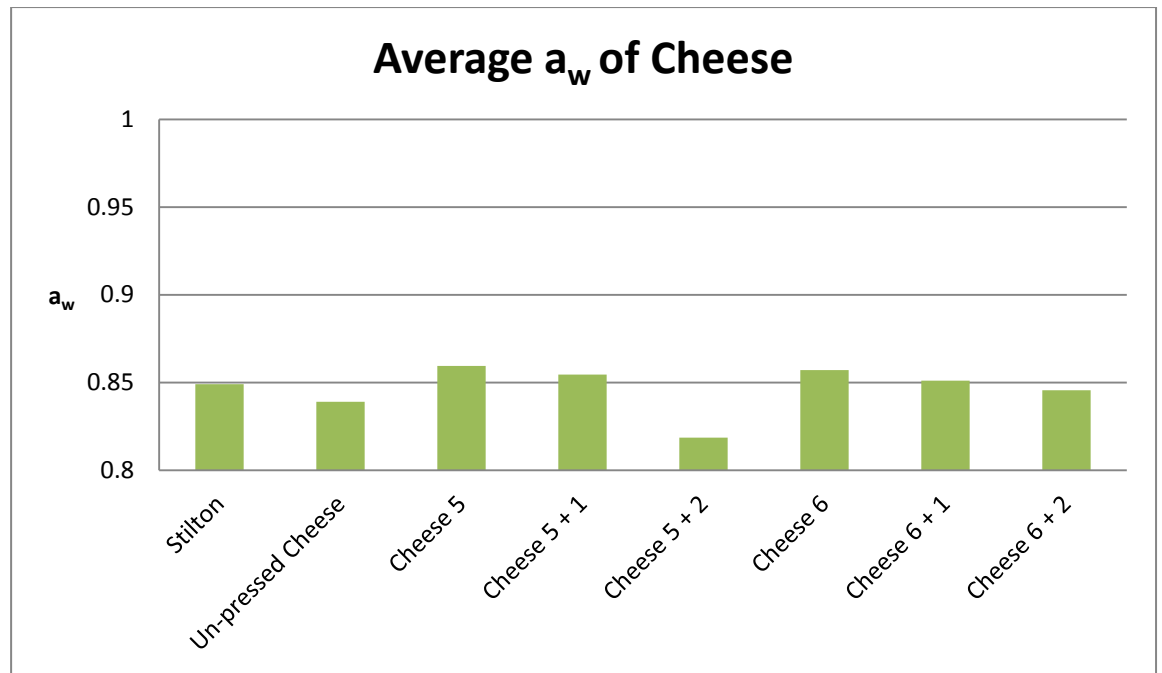


Figure 11: The average water activity of two pressed cheeses over three weeks of maturation compared to the un-pressed sample after 1 week of maturation and Stilton. Cheese 5 and 6 are after 1 week's maturation, cheese 5 + 1 and 6 + 1 is after 2 weeks' maturation and cheese 5 + 2 and 6 + 2 is after 3 weeks' maturation.

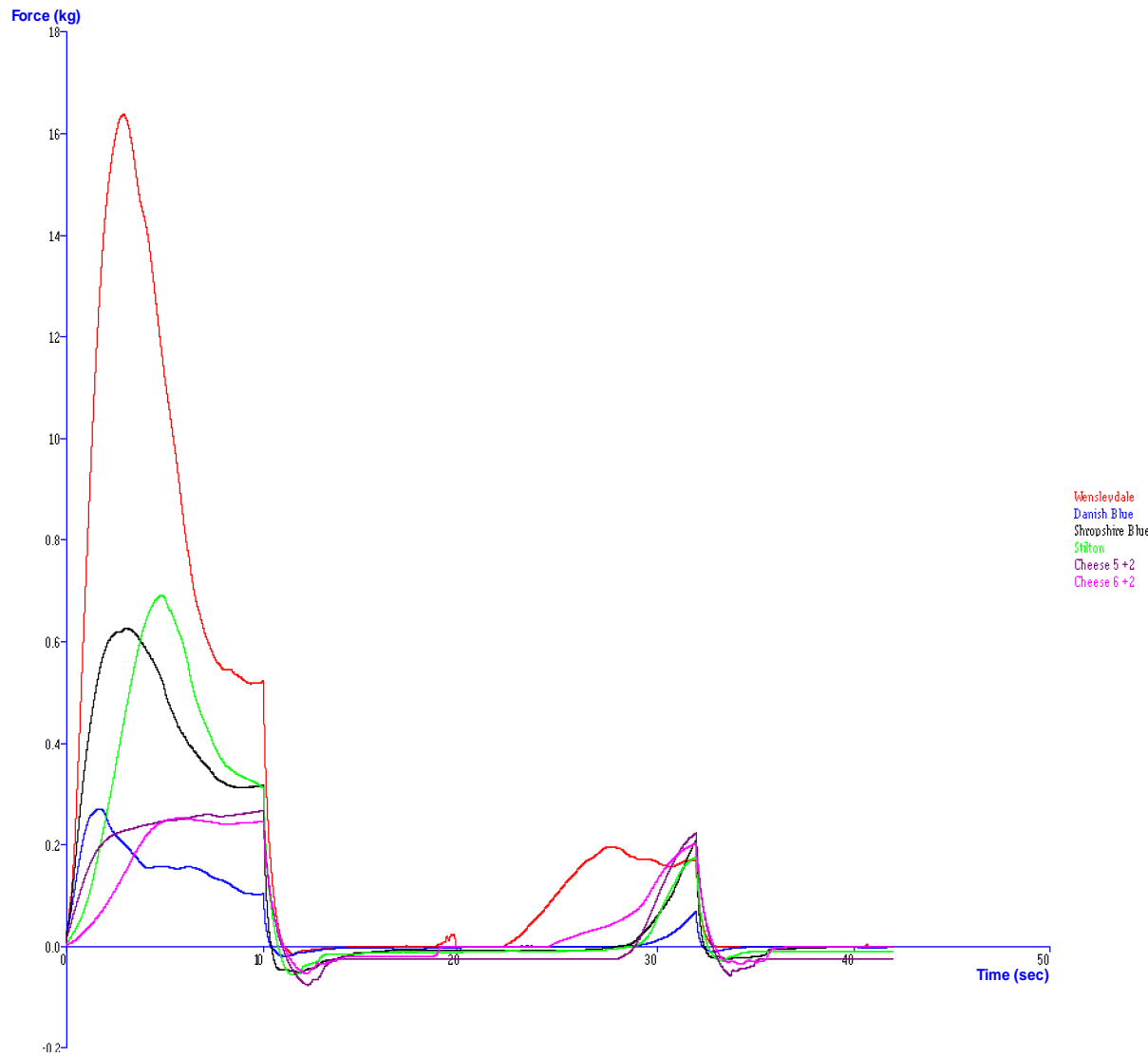


Figure 12: The texture of the two cheeses after 3 weeks of maturation compared to the commercial cheeses. Red = Wensleydale; Blue = Danish Blue; Black = Shropshire Blue; Green = Stilton; Purple = Sample 5 after 3 weeks of maturation; Pink = Sample 6 after 3 weeks of maturation.

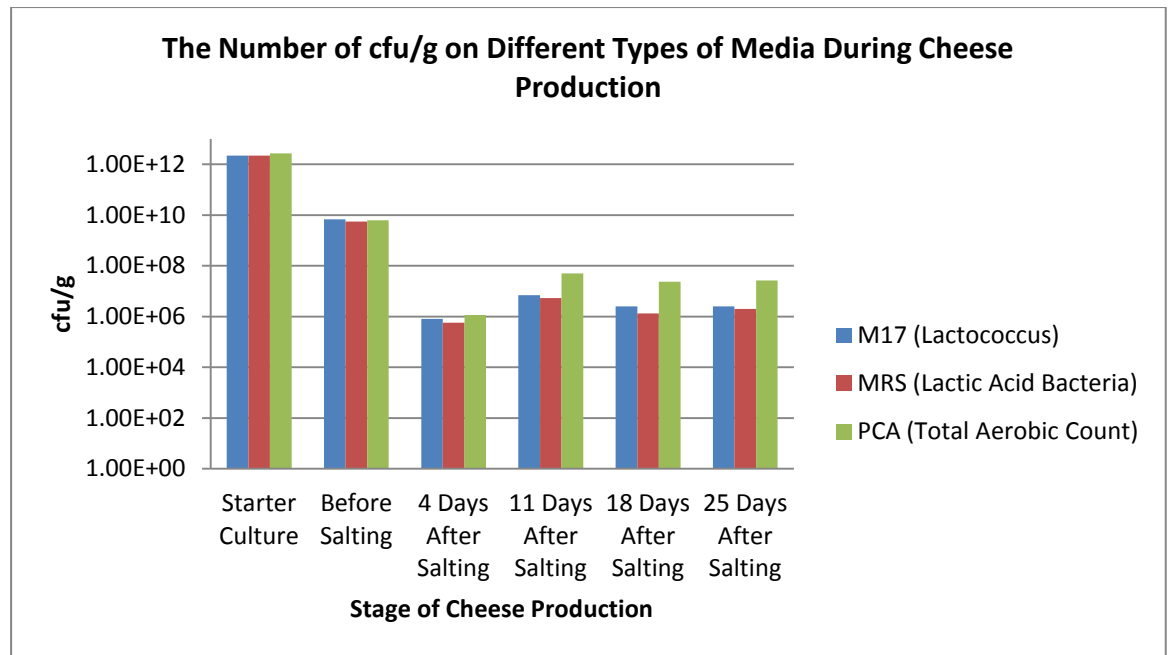


Figure 13: The number of cfu/g at different times during the cheese production process using three different types of media.

process. This means that the *Lactococcus* added as the starter culture survives the whole process without any other Lactic Acid bacteria contaminating the product (this would be shown by a higher number growing on the MRS agar). There is however some contamination. The PCA shows a higher count after salting, which means other aerobic microorganisms have contaminated the product. This is likely to be due to flora contamination as yeast was found to be growing on the RBC agar during the early stages of the process after salting, and mould growing on the RBC agar during the later stages. The salt used was a commercial salt bought at retail and therefore could have contaminants present including halophilic and alkaliphilic bacteria. These would survive during and after salting so would explain the fluctuation of the viable count post-salting (Ishikawa *et al.*, 2007). Alternatively these contaminants could come from the environment during cheese making as the product was not protected from the air.

3.1.9 FINAL SMALL-SCALE CHEESE MODEL

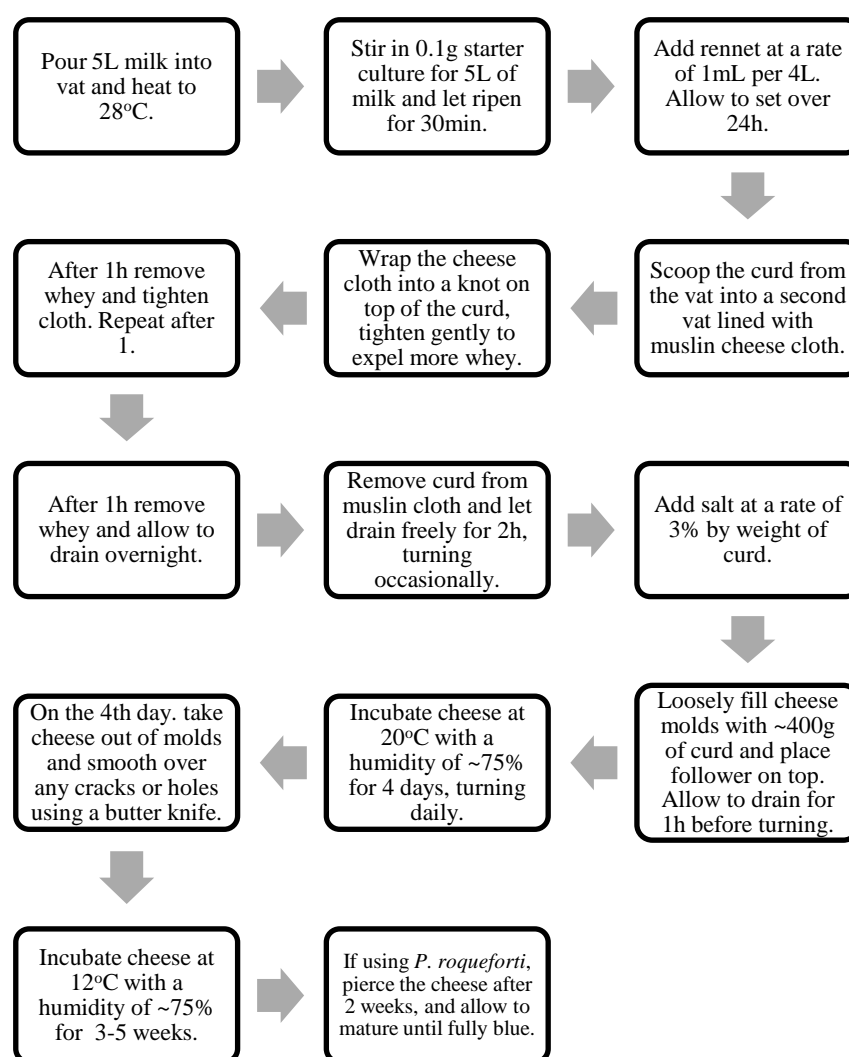


Figure 14: A final protocol to produce a small-scale Stilton cheese model.

Using this protocol, strains of *P. roqueforti* and yeast can be added to test for different flavour volatiles in the cheese.

3.2 SOLID PHASE MICROEXTRACTION GAS

CHROMATOGRAPHY-MASS SPECTROMETRY (SPME GC-MS)

3.2.1 MILK SAMPLES

In previous studies a simple milk model had been used to look at the flavour volatiles produced by individual cheese organisms (Gkatzionis, 2010). In this part of the study a comparison was made between using this milk model and using the developed cheese model to look at flavour volatile production to see if the more complex cheese matrix affected the interactions which took place. Using the milk model in Section 2.3, models were set up using a combination of microorganisms (Table 1). Sample D represents the same combination of strains as were used in the cheese production i.e. *P. roqueforti* and *Lactococcus lactis*. E, G and I show the impact of adding one particular yeast species, all of which had previously been isolated from Stilton cheese. Other combinations (C, F and H) show the interaction of each yeast with the *P. roqueforti* alone.

SPME GC-MS was carried out as in Section 2.6 and principal component analysis (PCA) was carried out on the results of the SPME GC-MS. This can be seen in Figure 15.

The results show that samples containing *P. roqueforti* with and without the *Lactococcus lactis* (B and D) produce high levels of ketones compared to the other samples. This is expected as the *P. roqueforti* is associated with ketone production which leads to the typical blue cheese aroma (Madkor *et al.*, 1987). The samples containing *Kluyveromyces lactis* Y12 (C and E) together with the *P. roqueforti* produced high levels of acids and esters and lower levels of ketones. This could be due to the metabolism of lactose and its related

<u>Sample</u>	<u>Inoculant</u>
A	Milk
B	Milk + SO17
C	Milk + SO17 + Y12
D	Milk + SO17 + <i>Lactococcus lactis</i>
E	Milk + SO17 + Y12 + <i>Lactococcus lactis</i>
F	Milk + SO17 + Y18
G	Milk + SO17 + Y18 + <i>Lactococcus lactis</i>
H	Milk + SO17 + SB15
I	Milk + SO17 + SB15 + <i>Lactococcus lactis</i>

Table 1: The different samples used for SPME GC-MS. SO17 = *P. roqueforti*; Y12 = *Kluyveromyces lactis*; Y18 = *Trichosporon beigleii*; SB15 = *Debaryomyces hansenii*.

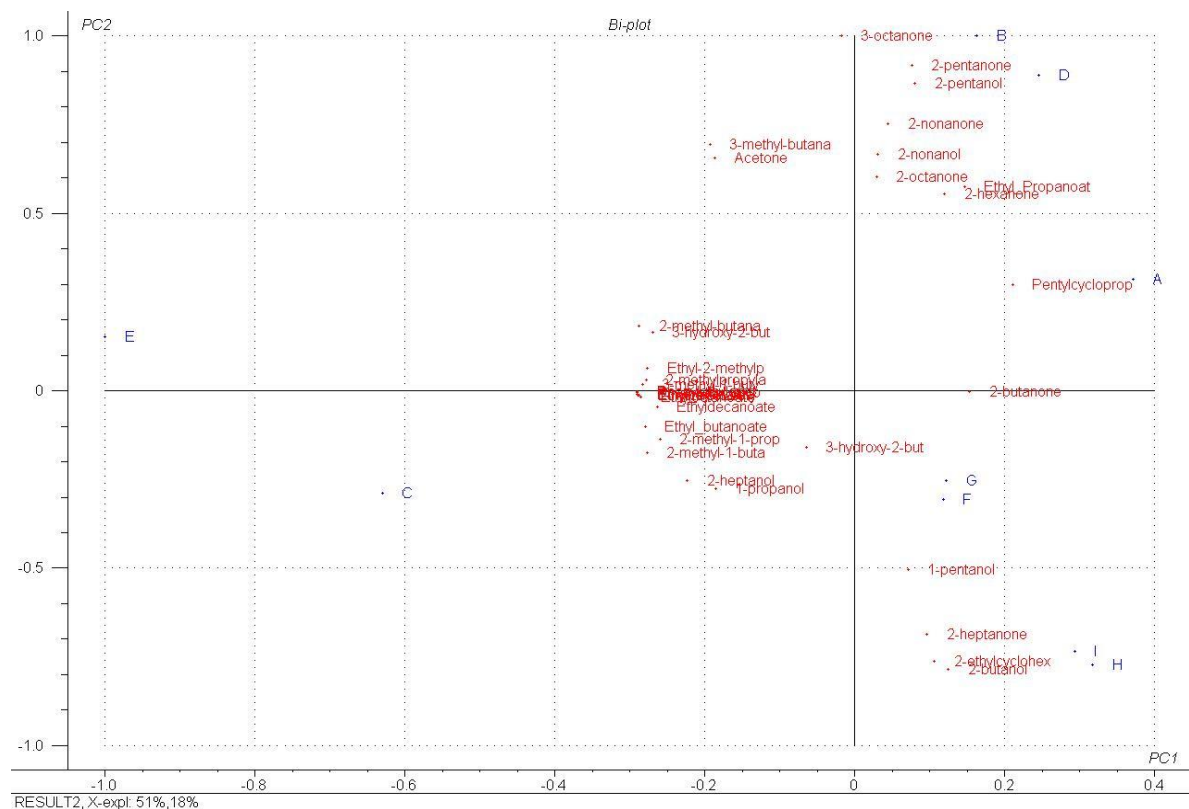


Figure 15: PCA of the milk samples. A = Milk; B = *P. roqueforti*; C = *P.*

roqueforti + *K. lactis*; D = *P. roqueforti* + *Lc. lactis*; E = *P. roqueforti* + *K.*

lactis + *Lc. lactis*; F = *P. roqueforti* + *T. beigleii*; G = *P. roqueforti* + *T.*

beigleii + *Lc. lactis*; H = *P. roqueforti* + *D. hansenii*; I = *P. roqueforti* + *D.*

hansenii + *Lc. lactis*.

pathways. *K. lactis* produces strong assimilation and fermentation of lactose (Roostita and Fleet, 1996) so when the lactose is metabolised, the metabolic pathway results in the production of these compounds.

The samples containing *Debaryomyces hansenii* (H and I) again produced less ketone compounds and more alcohols than the *P. roqueforti* on its own. This could be because the *D. hansenii* works in conjunction with the *P. roqueforti*. If the *P. roqueforti* metabolises triglycerides present in the milk (Blank and Privett, 1964), then the *D. hansenii* could metabolise the ketones produced into alcohols (Nakamura and Matsuda, 2002).

The samples containing *Trichosporon beigleii* (F and G) are closer to the centre of the PCA demonstrating these are not strongly associated with any particular compounds unlike the other yeast species and *P. roqueforti* samples. This species therefore seems to be either suppressing flavour volatile production by the *P. roqueforti* or producing all compounds equally.

3.2.2 CHEESE SAMPLES

A number of cheese models incorporating the yeast species were made.

However time constraints meant only one of these could be fully analysed for flavour volatile production. Because of its impact on *P. roqueforti*, the model incorporating *Trichosporon beigleii* was the one chosen to examine in detail.

Three cheese samples were made using the method in Section 3.1.9; all samples contained the mixed *Lactococcus* starter culture but one sample was inoculated with 0.12mL of a commercial *P. roqueforti* (Orchard Valley Dairy Supplies), a second sample was inoculated with 0.12mL of a commercial *P. roqueforti* and 1mL of solution containing $\sim 10^8$ cells/mL of *T. beigleii* with the third sample containing the starter culture alone. The protocol was

followed for 10 days (4 days into the ripening stage) after the inoculation of the milk so as to show a comparison between the milk and cheese models when run through the SPME GC-MS method.

Figure 16 shows two cheeses with added inoculants after 10 days. The sample with the *P. roqueforti* was engulfed by the mould after 10 days and when a cross section was cut, a clear crust of the *P. roqueforti* on the surface only could be seen (Figure 17). This demonstrates the organism only grows and sporulates where there is high oxygen content. In contrast when *T. beigleii* was added there was a major suppression of the *P. roqueforti* growth.

Samples were taken from the inner core and the surface ('crust') of each cheese sample and run through the SPME GC-MS; the results were analysed by PCA (Figure 18). The white cheeses with the starter culture alone (J and K) are grouped together with the esters and alcohols. This is because the cheese sample only contains *Lc.* species which metabolise the lactose. The metabolic pathway this follows involves the production and breakdown of glucose and pyruvate which leads to the final production of alcohols and esters. The core and the crust gave very similar results showing the flora is not really different at the surface.

The inner part and the crust of the *P. roqueforti* inoculated cheese gave very different results. Surprisingly the inner core (M) gave higher ketone levels than the outer crust (L). The crust of the cheese containing the *P. roqueforti* is grouped with some alcohols. The difference between the inner and outer parts of this sample could be due to the greater growth of *P. roqueforti* on the surface. The high growth levels could have led to an increase in the rate of metabolism of the triglycerides in the cheese. Although ketones are the



Figure 16: The cheese on the left was inoculated with *P. roqueforti* and *T. beigleii* and shows little sign of *P. roqueforti* growth. The cheese on the right was inoculated with *P. roqueforti* and has been engulfed by the mould.



Figure 17: A cross section of the cheese inoculated with *P. roqueforti*. This shows a clear crust formed by the mould.

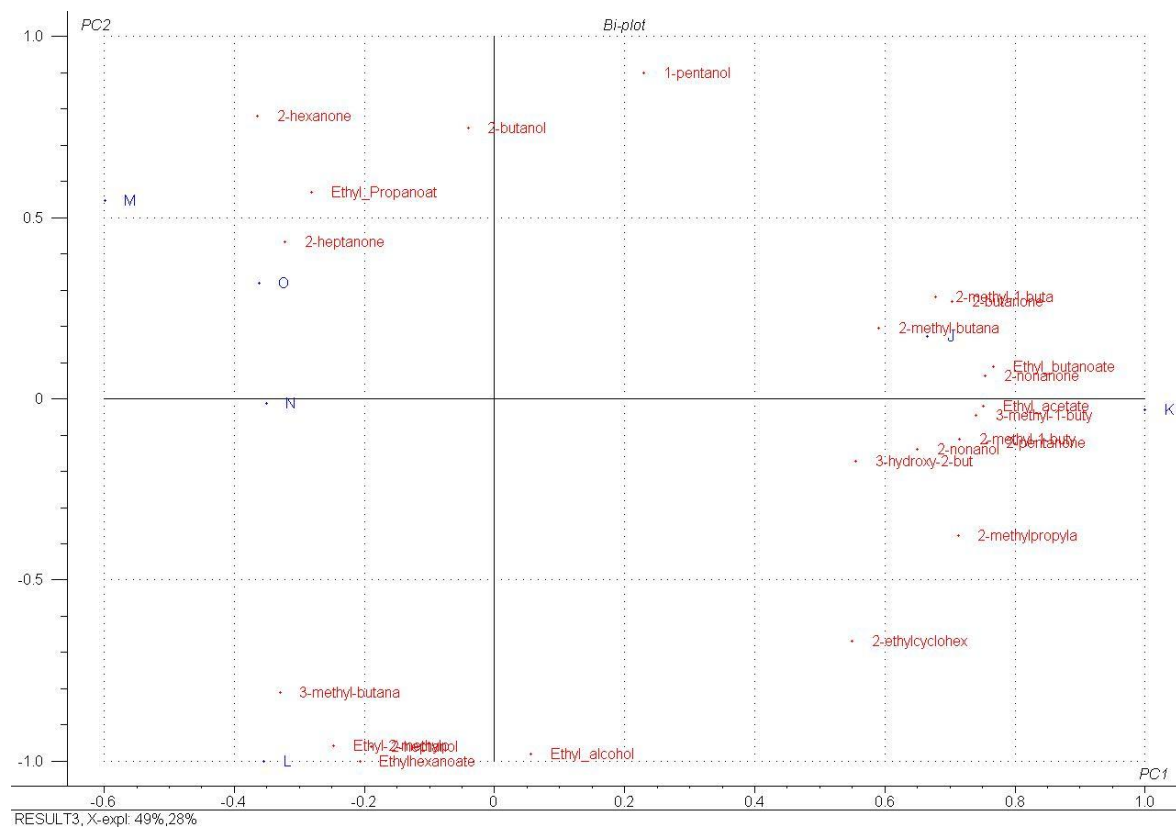


Figure 18: PCA of the cheese samples. J = White cheese crust; K = White cheese inner; L = *P. roqueforti* cheese crust; M = *P. roqueforti* cheese inner; N = *P. roqueforti* + *T. beigleii* cheese crust; O = *P. roqueforti* + *T. beigleii* cheese inner.

primary product of this metabolism pathway, overproduction could lead to continued metabolism and the production of secondary alcohols by ketone metabolism.

The crust of the cheese inoculated with the *T. beigleii* (N) shows no grouping with any compounds. This confirms what was seen in the milk model and demonstrates the effect of the yeast inhibiting the growth of *P. roqueforti* hence the lack of flavour compounds produced.

The inside of the two cheese samples inoculated with *P. roqueforti* with or without *T. beigleii* are grouped together (O and M respectively) with ketone production. Although *P. roqueforti* does not sporulate inside the cheese (i.e. does not go blue), it is clearly metabolising and producing ketones. In this situation the *T. beigleii* does not appear to be influencing the ketone production as significantly as the crust of the same sample. The inside of the cheese has less oxygen and although yeasts are facultative anaerobes, they will grow better in the presence of oxygen – it may be that this is why a difference is seen in the way it influences the *P. roqueforti* growth and volatile production on the surface.

3.2.3 COMPARISON OF MILK AND CHEESE SAMPLES

The cheese samples and the milk samples were compared using PCA (Figure 19). There are higher levels of ketones, for example 2-butanone, which are produced by the milk samples (B and D) than any of the cheese samples, even those inoculated with the *P. roqueforti* alone (L and M). This could be because the cheese matrix may provide a physical barrier to the release of compounds. Also, the higher fat content of the cheese will act as a solvent for lipophilic volatile compounds.

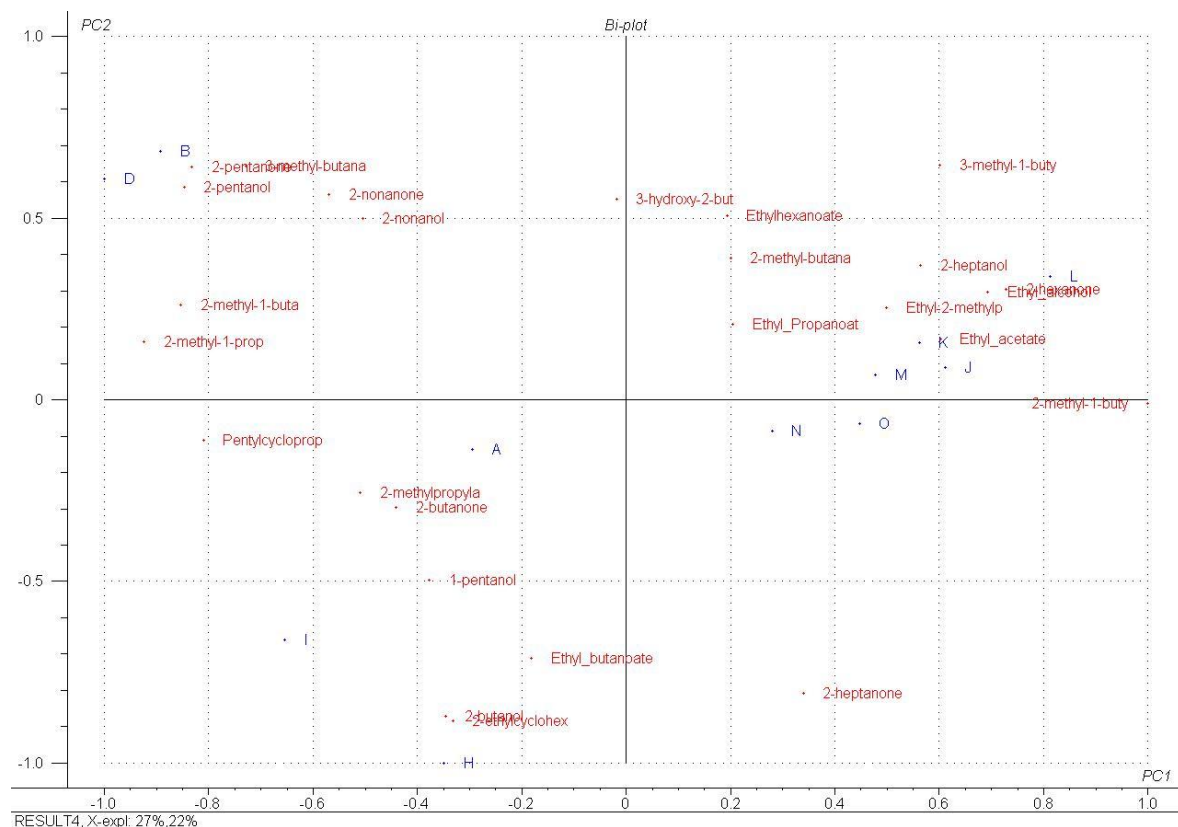


Figure 19: A PCA showing a comparison between the milk and cheese samples. Milk models: A = Milk; B = *P. roqueforti*; D = *P. roqueforti* + *Lc. lactis*; H = *P. roqueforti* + *D. hansenii*; I = *P. roqueforti* + *D. hansenii* + *Lc. lactis*. Cheese models: J = White cheese crust; K = White cheese inner; L = *P. roqueforti* cheese crust; M = *P. roqueforti* cheese inner; N = *P. roqueforti* + *T. beigleii* cheese crust; O = *P. roqueforti* + *T. beigleii* cheese inner.

Both the cheese and milk samples produce alcohols, although they have produced different amounts of the different alcohols. The milk models containing *T. beigleii* (H and I) appear to have produced more 1-Pentanol where as the cheese samples have produced more 2-Heptanol and the milk samples containing *P. roqueforti* (D and B) have produced more 2-Pentanol (Figure 20). 2-Pentanol and 2-Heptanol are both secondary alcohols and are produced during the metabolism of triglycerides by *P. roqueforti*, which would provide an explanation for why they are produced less by samples containing *T. beigleii*. The 1-Pentanol is a primary alcohol and is produced by the metabolism of casein, possibly by the lactic acid bacteria (Stiles and Holzapfel, 1997), although it may be inhibited in other samples by the *P. roqueforti*.

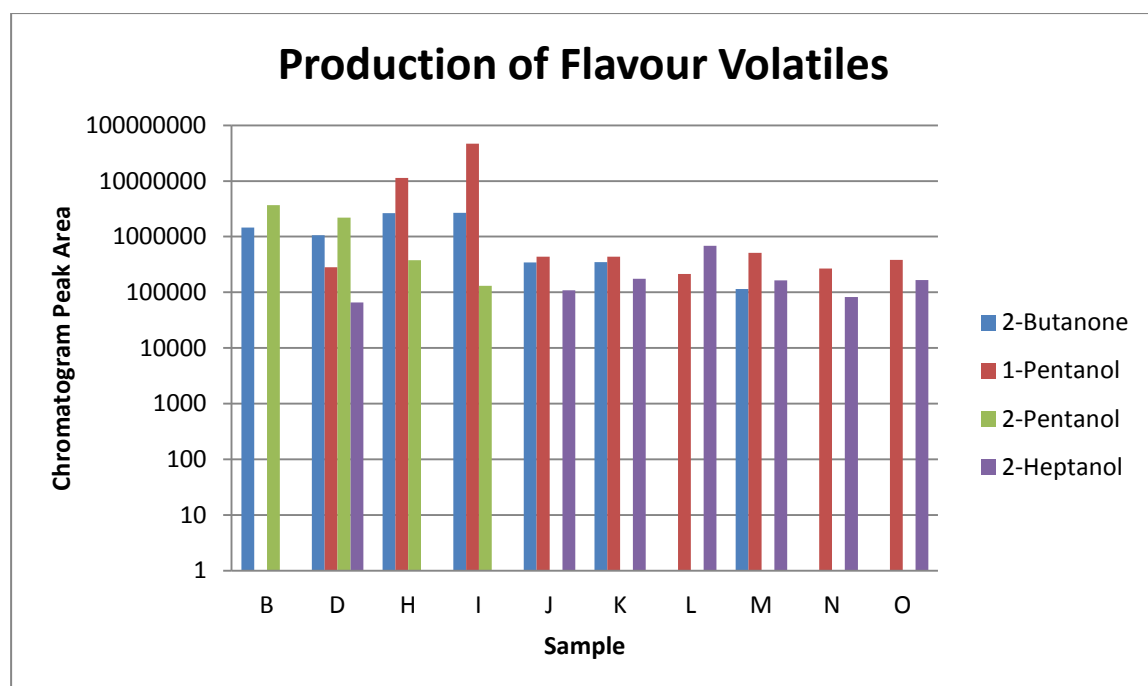


Figure 20: A graph showing a comparison in production of some flavour volatiles from the milk and cheese samples. Milk models: B = *P. roqueforti*; D = *P. roqueforti* + *Lc. lactis*; H = *P. roqueforti* + *D. hansenii*; I = *P. roqueforti* + *D. hansenii* + *Lc. lactis*. Cheese models: J = White cheese crust; K = White cheese inner; L = *P. roqueforti* cheese crust; M = *P. roqueforti* cheese inner; N = *P. roqueforti* + *T. beigleii* cheese crust; O = *P. roqueforti* + *T. beigleii* cheese inner.

4 DISCUSSION

The aim of this study was to develop a protocol for a small-scale cheese model without the use of external contaminants (such as yeast) that could be used to examine the impact that different organisms have on the flavour development of blue cheese using a controlled flora. The protocol was developed as far as possible within the time constraints of the project. The final product was developed to produce a cheese which has the water activity and some of the typical flavour volatiles of blue cheeses like Stilton, although further work could be carried out to develop this further. The texture analysis results showed that a gradual increase in hardness was achieved through a number of modifications to the method, and the water activity of the cheese was equal to that of commercial Stilton.

Although a final small-scale cheese model has been produced, the cheese is different to commercial Stilton. The changes made to the original Stichelton protocol (Figure 1) have been quite significant and some of them were necessary possibly because of the lack of external flora. In particular no true crust developed, but this has been shown to need the presence of yeasts and bacteria not added commercially to the cheese. The addition of more microorganisms, such as the yeasts associated with the Stilton crust or even bacteria such as *Lactobacillus* which are present in the mature commercial product (Ercolini *et al.*, 2003) may start to affect the model produced. This highlights the importance of the microbial flora which is introduced into the cheese making process through machine or human intermediates and has an impact on the final texture, aroma and flavour of Stilton cheese.

The results of the SPME GC-MS analysis have concluded that although not a finished product, the small-scale model can be used to study the development of the flavour and aroma of Stilton cheese. Different flavour compounds were produced by the cheese samples when compared to the milk model samples, and show a difference between cheese containing different microorganisms.

The results of the SPME GC-MS have confirmed that the environmental yeast contaminants do contribute to the different flavours that can be found between different varieties of blue cheese and even between different batches of the same cheese.

During cheese ripening, the formation of flavours involves various chemical and biochemical conversions of milk components. There are three main metabolic pathways involved which are; the conversions of lactose (glycolysis), fat (lipolysis) and caseins (proteolysis). The starter culture containing *Lactococcus* and *P. roqueforti* is the main source of the enzymes involved in these pathways (Smit *et al.*, 2005) although contaminants have also been known to provide enzymes for these (Peterson and Marshall, 1990).

The metabolism of lactose leads to the production of ethanol, acetate and other aldehydes and acids which give off a typical yoghurt smell. These would be prominent in early fermentation stage of the cheese making process before fungal microorganisms begin to grow. This is primarily due to the lactic acid bacteria added as a starter. Figure 17 shows this as the milk samples containing *Lactococcus lactis* and *P. roqueforti* contain high levels of Ethylacetate and the cheese samples containing only *Lc. lactis* as a starter in Figure 18 contain high levels of acetone (Smit *et al.*, 2005).

During glycolysis, lactic acid is produced which is utilised by *P. roqueforti*.

The lactic acid is produced by the *Lactococcus lactis* in the centre of the cheese, and the *P. roqueforti* is a surface-ripening mould, so cannot access it.

Surface flora produces ammonia. This ammonia enters the cheese and in doing so aids in the diffusion of lactic acid to the surface of the cheese. Here, the lactic acid can be metabolised by the *P. roqueforti* (Lawrence *et al.*, 1987).

The lactic acid is broken down and the primary products are ketones.

The lipolysis results in the formation of ketones, esters and secondary alcohols. Lactic acid bacteria contribute little to this metabolic stage but the surface ripening *P. roqueforti* are highly active in fat conversion and produce the flavours and smells typically associated with blue cheese (Molimard and Spinnler, 1996). Figure 17 again shows that the milk samples containing the *P. roqueforti* contain high levels of these 'blue note' ketones and secondary alcohols.

The break down of caseins during proteolysis is highly important during the production of flavour volatiles. Rennet enzymes and cell-envelope proteinase and peptidases from lactic acid bacteria breakdown the caseins into small peptides and amino acids and continued metabolism leads to various alcohols, aldehydes, acids and esters (Smit *et al.*, 2005). More of these flavour volatiles were found in all of the cheese samples compared to the milk samples (Figure 19) as they contained the enzymes found in rennet.

The use of salt stops the acidification of the curds and the effectiveness of the lactic acid bacteria (Kets *et al.*, 1996). Figure 2 shows that the milk is acidified quickly by the production of lactic acid by the *Lactococcus lactis*, however when the salt is added to the curd, the acidification stops. This can be seen in

Figure 13 where the number of lactic acid bacteria drops significantly after salting. This could lead to an increase in the activity of the mould because until this point, there were no visual signs that *P. roqueforti* was present. As the *P. roqueforti* can metabolise the lactic acid, if the acid production is slowed, then it gives the *P. roqueforti* a chance to begin its metabolism and form the ketones (McGugan *et al.*, 1979).

The final protocol (Figure 14) uses a lot more of the starter culture than the protocol from Stichelton (Figure 1). This is because Stichelton uses raw milk compared to the UHT milk used in this study and by Stilton producers. Cheese producers who use raw milk typically use less starter culture than those who use UHT or pasteurised milk. This is because when using raw milk, natural flora present in the milk such as *Lactobacillus curvatus* and *Lactobacillus fermentum* aid in the fermentation of the milk (Somers *et al.*, 2001). Using less of a commercial starter culture allows the natural flora to survive and in doing so other products are formed from their metabolic pathways to give each cheese a distinct flavour (Estrella *et al.*, 2002). This may be why the first few attempts during this study did not lead to the milk fermenting to producing curds and whey. Using this information, a number of different microorganisms can be added as a starter to determine the effect the non-starter flora has on the flavour volatiles produced.

Throughout this study, no piercing was involved due to the overgrowth of *P. roqueforti*. After the main study was complete, a cheese was pierced after being engulfed by the mould and a cross section revealed blue veining. The growth of *P. roqueforti* could have been controlled by reducing the amount of oxygen reaching the surface of the cheese during ripening. A way in which

commercial Stilton producers do this is to wrap the Stilton in film stopping the *P. roqueforti* from growing until the piercing stage, by which point environmental yeast contaminants have formed a crust on the surface, inhibiting the growth of the *P. roqueforti* (LCD, 2012). This could therefore be applied to the small-scale cheese model to see what effect it has on the *P. roqueforti* and whether it is possible for blue veining to occur without the whole cheese being engulfed.

The conditions used for cheese making were controlled throughout the study to minimise contamination. However this did not stop all contamination as Figure 13 shows that as the cheese ripened, the number of bacteria was higher on the PCA plates than on the M17 and MRS agars. PCA allows the growth of aerobic microorganisms whereas M17 and MRS only allows the growth of *Lactococcus* and lactic acid bacteria respectively, so non-starter organisms which were not lactic acid bacteria were present. The PCA count was only higher after salting. It has been shown that commercial salt can carry yeast species and bacteria which could contaminate cheese (Guinee and Fox, 1999). However, it is not just through the salt that contamination can occur. Fungal spores can spread and contaminate through the air (Hocking and Faedo, 1992) and although conditions were controlled during this study, bacterial contamination can occur through machine or human contact during the commercial process (Jacquet *et al.*, 1993). This shows that even using UHT or pasteurised milk for the production of Stilton, non-starter microorganisms can still contaminate the cheese.

The *P. roqueforti* strain used in the milk model was isolated from commercial Stilton; however the strain used in the cheese models was bought from dairy

suppliers. This could have led to differences in their metabolic pathways and therefore the flavour volatiles were produced. If time had allowed, I would have liked to study the differences between the two strains to determine their effects within the milk and cheese models.

This study has shown that it is possible to make a small-scale blue cheese model following a similar process to the commercial production of Stilton and Stichelton. From this I have been able to see the effects on flavour and aroma by different microorganisms. The study has also demonstrated how the starter culture acidifies the milk during fermentation and how the ripening is affected by different surface fungi. This model could be used by commercial blue cheese makers to develop the flavour and aroma of their cheese.

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APPENDIX 1: MEDIA AND CHEMICALS

A. MEDIA

1. BRAIN HEART INFUSION AGAR (Oxoid code CM1136)

23.5g was dissolved in 500mL of RO water and autoclaved at 121°C for 15 minutes. The media was allowed to cool to 55°C before plating. Plates were stored at 4°C.

2. M17 AGAR (Oxoid code CM0785)

24.1g was dissolved in 475mL of RO water and autoclaved at 121°C for 15 minutes. The media was allowed to cool to 50°C before the addition of 25mL 10% (w/v) sterile lactose solution (Oxoid code LP00070). This was mixed before plating. Plates were stored at 4°C

3. MRS AGAR (Oxoid code CM0361)

31g was dissolved in 500mL of RO water and autoclaved at 121°C for 15 minutes. The media was allowed to cool to 55°C before plating. Plates were stored at 4°C.

4. ROSE BENGAL CHLORAMPHENICOL AGAR (Oxoid code CM0549)

16g was dissolved in 500mL of RO water. The media was brought to the boil before the addition of one reconstituted vial of Chloramphenicol supplement (Oxoid code SR0078E). This was mixed and autoclaved at 121°C for 5 minutes. The media was allowed to cool before plating. Plates were stored at 4°C.

5. PLATE COUNT AGAR (Oxoid code CM0463)

11.75g was dissolved in 500mL of RO water and autoclaved at 121°C for 15 minutes. The media was allowed to cool to 55°C before plating. Plates were stored at 4°C.

6. MRS Broth (Oxoid code CM0359)

26g was dissolved in 500mL of RO water and autoclaved at 121°C for 15 minutes. The broth was stored at 4°C.

B. CHEMICALS

1. PHOSPHATE BUFFERED SALINE (Oxoid code BR0014)

5 tablets were dissolved in 500mL of RO water and autoclaved at 115°C for 10 minutes. This was then stored at room temperature.

2. MAXIMUM RECOVERY DILUENT (Oxoid code CM0733)

4.75g was dissolved in 500mL of RO water and autoclaved at 121°C for 15 minutes. This was then stored at room temperature.

3. ¼ STRENGTH RINGERS SOLUTION (Oxoid code BR0052)

1 tablet was dissolved in 500mL of RO water and autoclaved at 121°C for 15 minutes. This was then stored at room temperature.

APPENDIX 3: VIABLE COUNT

A) 1mL milk: No growth on any media.

B) 0.1g starter culture: No growth on RBC.

[illegible]

C) 1g cheese before salting: Little yeast growth on RBC.

[illegible]

D) 1g cheese 4 days after salting: Moderate yeast growth on RBC.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
M17	-	-	17	0	0	0	0	0	0
	-	-	15	0	0	0	0	0	0
	-	-	17	4	1	0	0	0	0
MRS	-	-	8	4	0	0	0	0	0
	-	-	8	0	1	0	0	0	0
	-	-	18	1	0	0	0	0	0
PCA	-	-	25	3	0	0	0	0	0
	-	-	21	3	0	0	0	0	0
	-	-	23	3	3	0	0	0	0

E) 1g cheese 11 days after salting: High yeast growth on RBC.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
M17	-	-	-	17	0	0	0	0	0
	-	-	-	14	1	0	0	0	0
	-	-	-	11	2	1	0	0	0
MRS	-	-	-	13	0	1	0	0	0
	-	-	-	9	1	0	0	0	0
	-	-	-	10	0	0	0	0	0
PCA	-	-	-	33	10	1	0	0	0
	-	-	-	35	9	0	0	0	0
	-	-	-	28	11	0	0	0	0

F) 1g cheese 18 days after salting: Moderate yeast growth and little mould growth on RBC.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
M17	-	-	34	4	0	0	0	0	0
	-	-	22	8	0	0	0	0	0
	-	-	36	3	0	0	0	0	0
MRS	-	-	31	3	0	0	0	0	0
	-	-	39	2	0	0	0	0	0
	-	-	29	3	0	0	0	0	0
PCA	-	-	-	-	5	0	0	0	0
	-	-	-	-	3	0	0	0	0
	-	-	-	-	6	0	0	0	0

G) 1g cheese 25 days after salting: Little yeast growth and high mould growth on RBC.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
M17	-	-	39	4	1	0	0	0	0
	-	-	36	6	1	0	0	0	0
	-	-	31	5	0	0	0	0	0
MRS	-	-	36	4	0	0	0	0	0
	-	-	34	5	1	0	0	0	0
	-	-	39	3	0	0	0	0	0
PCA	-	-	-	29	3	0	0	0	0
	-	-	-	39	6	0	0	0	0
	-	-	-	32	7	0	0	0	0